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(54) Title: GENOMIC SCREEN FOR EPIGENETICALLY SILENCED TUMOR SUPPRESSOR GENES

(57) Abstract: Methods of genomic screening to identify epigenetically silenced genes, including epigenetically silenced tumor suppressor genes are provided. Also provided are methods of detecting a cancer, for example, an esophageal squamous cell carcinoma or a head and neck squamous cell carcinoma, as are methods of treating a subject having such a cancer.

## **GENOMIC SCREEN FOR EPIGENETICALLY SILENCED TUMOR SUPPRESSOR GENES**

[0001] This application claims the benefit of priority under 35 U.S.C. § 119(e)(1) of U.S. Serial No. 60/362,577, filed March 7, 2002, the entire content of which is incorporated herein by reference.

[0002] This invention was made in part with government support under Grant No. CA84986-04 awarded by the National Institutes of Health. The United States government may have certain rights in this invention.

### **BACKGROUND OF THE INVENTION**

#### **FIELD OF THE INVENTION**

[0003] The invention relates generally to methods of detecting tumor suppressor genes that are epigenetically silenced in cancer cells, and more specifically to methods for diagnosing a cancer such as esophageal cancer and head and neck cancer, and to methods of treating such cancers.

#### **BACKGROUND INFORMATION**

[0004] Although cancers generally are considered to be due to genetic changes such as mutations of a gene, it has become clear that epigenetic mechanisms, which do not result in mutations of the DNA sequence, also can result in cancers. The most commonly observed epigenetic change involves silencing of gene expression due to methylation of the gene sequence, particularly the 5' upstream gene regulatory sequences. Methylation of cytosine residues located 5' to guanine in CpG dinucleotides, particularly in CpG-rich regions (CpG islands), often is involved in the normal regulation of gene expression in higher eukaryotes. For example, extensive methylation of CpG islands is associated with transcriptional inactivation of selected imprinted genes; as well as the genes on the inactivated X chromosome in females. Aberrant methylation of normally unmethylated CpG islands also has been found in immortalized and transformed cells, and has been associated with transcriptional inactivation of defined tumor suppressor genes in human cancers.

[0005] Changes to genes that are associated with cancer, including mutations that result in loss of expression of gene or expression of a defective gene product, and epigenetic mechanisms such as methylation-silencing of gene transcription, provide markers useful for determining whether a cell is susceptible to loss of normal growth control and, therefore, potentially a cancer cell. For example, a mutation of the BRCA1 gene has been associated with breast cancer. As such, diagnostic tests can be performed using cells, for example, from a woman with a family history of breast cancer to determine whether the woman has the BRCA1 mutation that is a marker for breast cancer. The prostate specific antigen (PSA) is another example of a marker, in this case for prostate cancer. Although neither the defect resulting in expression of the PSA nor the normal function of PSA in the body is known, PSA nevertheless provides a valuable cancer marker because it allows the identification of men predisposed to prostate cancer or at a very early stage of the disease such that effective therapy can be implemented. More recently, methylation-silenced transcription of a suppressor of cytokine signaling/cytokine-inducible SH2 protein family member, the SOCS-1 gene was found in various cancers, including hepatocellular carcinoma, multiple myeloma, and acute leukemias. As such, screening assays directed to detecting the methylation status of the SOCS-1 gene can provide diagnostic information relating to such cancer.

[0006] As cancer often is a silent disease that does not present clinical signs or symptoms until the disease is well advanced, the availability and use of markers that allow the identification of individuals susceptible to a cancer, or even that allow detection of a cancer at an early stage, can be of great benefit. Unfortunately, such markers are not available for most cancers. As such, many cancer patients do not seek medical assistance until the cancer is at a stage that requires radical therapy, or is untreatable. Thus, a need exists for markers that can be used to detect cancer cells. The present invention satisfies this need and provides additional advantages.

#### SUMMARY OF THE INVENTION

[0007] The present invention relates to a method of identifying at least one epigenetically silenced gene associated with a cancer. Such a method can be performed, for example, by contacting an array of nucleotide sequences representative of a genome with

nucleic acid molecules corresponding to RNA expressed in cancer cells contacted with at least one agent that reactivates expression of epigenetically silenced genes, under conditions suitable for selective hybridization of nucleic acid molecules to complementary nucleotide sequences of the array; and detecting increased hybridization of nucleic acid molecules of the cancer cells contacted with the agent(s) to a subpopulation of nucleotide sequences of the array as compared to a level of hybridization, if any, of nucleic acid molecules corresponding to RNA expressed in the cancer cells to at least one nucleotide sequence of the subpopulation of nucleotide sequences, under said conditions, whereby increased selective hybridization identifies reactivated expression of an epigenetically silenced gene.

[0008] The nucleic acid molecules corresponding to RNA that are contacted with the nucleotide sequences of the array can be DNA or RNA, including, for example, cDNA, rRNA, mRNA, or any other nucleic acid molecules representative of RNA expressed in a cell. The agent that reactivates expression of epigenetically silenced genes can be a demethylating agent such as a methyltransferase inhibitor (e.g., 5-aza-2'-deoxycytidine; 5Aza-dC), a histone deacetylase inhibitor (e.g., trichostatin A; TSA), or a combination thereof. The cancer cells can be cells of a sarcoma or a carcinoma, for example, esophageal cancer cells.

[0009] In one embodiment, the method comprises contacting the array with nucleic acid molecules corresponding to RNA expressed in cancer cells contacted with 5Aza-dC, TSA, or a combination thereof. For example, where the cancer cells are esophageal squamous cell carcinoma (ESCC) cells, the epigenetically silenced gene identified by the method can be a gene as listed in Table 2, or a combination of such genes. Where the cancer cells are head and neck squamous cell carcinoma (HNSCC) cells, the epigenetically silenced gene identified by the method can be a gene as listed in Table 5 or Table 6. In one aspect of this embodiment, the cancer is ESCC, and the epigenetically silenced gene is an apolipoprotein D (ApoD) gene, neuromedin U (NU) gene, swisprosin-2 gene, Hep27 gene, KIF5C gene, keratin 14 gene, transglutaminase 2 gene, MUC1 gene, interleukin-1 receptor 2 (IL-1 R2) gene, crystallin alpha2 gene, cysteine-rich protein with LIM (CRIP-1) gene, Rad gene, HEM45 gene, KLF6 gene, follistatin related protein FLRG gene, XAP-5 gene, Tbc1d1 gene, cyclin G1 interacting protein gene, or a combination thereof. In another

aspect of this embodiment, the epigenetically silenced gene is an ApoD gene, NU gene, swisprosin-2 gene, cytokine-like factor-1 (CLF-1) gene, CRIP-1 gene, cellular retinol binding protein (CRBP) gene, metallothionein 1G gene, keratin 14 gene, IL-1 R2 gene, crystallin alpha2 gene, or a combination thereof.

[0010] In another embodiment, at least one epigenetically silenced gene identified according to a method of the invention is a methylation silenced gene. For example, the methylation silenced gene in an ESCC can be an ApoD, NU, CLF-1, CRIP-1, claudin-3, uncoupling protein-2, metallothionein 1G, transglutaminase 2, or apolipoprotein C1 gene, or a combination thereof. In another embodiment, the epigenetically silenced gene is a tumor suppressor gene. In one aspect, the tumor suppressor gene is an ApoD gene, a NU gene, or a CRIP-1 gene, each of which, as disclosed herein, exhibits tumor suppressor activity. In another aspect, the tumor suppressor gene is a neuromedin B gene, or a receptor of G protein signaling 2 (RGS2) gene.

[0011] The present invention also relates to a method for identifying a cell that exhibits or is predisposed to exhibiting unregulated growth. Such a method can be performed, for example, by detecting, in a test cell, epigenetic silencing of at least one gene as set forth in Table 2, or a combination of such genes, for example, an ApoD, NU, swisprosin-2, Hep27, KIF5C, keratin 14, transglutaminase 2, MUC1, IL-1 R2, crystallin alpha2, CLF-1, CRIP-1, Rad, HEM45, KLF6, follistatin related protein FLRG, XAP-5, Tbc1d1, cyclin G1 interacting protein, CRBP, metallothionein 1G, claudin-3, uncoupling protein-2, or apolipoprotein C1 gene, or a combination thereof.

[0012] The test cell, is a cell exhibiting, or predisposed to exhibiting unregulated growth, can be a neoplastic cell, for example, a premalignant cell or a malignant cell (i.e., a cancer cell). As such, the cell can be a cell known or suspected of being a carcinoma cell, a sarcoma cell, or the like. In one embodiment, the cell exhibiting or predisposed to exhibiting unregulated growth, or suspected of being such a cell is a cancer cell. In one aspect of this embodiment, the cancer cell is an ESCC cell, or a cell suspected of being an ESCC cell. In another aspect of this embodiment, the cancer cell is an HNSCC cell, or a cell suspected of being an HNSCC cell.

[0013] In one embodiment, the epigenetic silencing comprises methylation silencing, wherein the method can be practiced by detecting methylation and/or methylation silencing of one or more genes in the test cell. In one aspect of this embodiment, methylation silencing is detected by contacting a region comprising a 5' regulatory region of the nucleic acid molecule comprising the gene with a methylation sensitive restriction endonuclease, which cleaves a recognition site in the 5' regulatory region containing a methylated cytosine residue of a CpG dinucleotide, whereby cleavage of the nucleic acid molecule is indicative of methylation silencing of the gene of the test cell. A methylation sensitive restriction endonuclease useful for such a method can be, for example, Acc III, Ban I, BstN I, Msp I, or Xma I. In another aspect of this embodiment, methylation silencing is detected by contacting a region comprising a 5' regulatory region of the nucleic acid molecule comprising the gene with a methylation sensitive restriction endonuclease, which cleaves a recognition site in the 5' regulatory region comprising a methylated cytosine residue of a CpG dinucleotide, provided the cytosine residue of the CpG dinucleotide is unmethylated, whereby a lack of cleavage of the nucleic acid molecule is indicative of methylation silencing of the gene of the test cell. A methylation sensitive restriction endonuclease useful for such a method can be, for example, Acc II, Ava I, BssH II, BstU I, Hpa II, or Not I.

[0014] Methylation silencing of gene expression also can be detected by contacting a 5' regulatory region of a nucleic acid molecule comprising the gene of a test cell with a chemical reagent that selectively modifies either an unmethylated cytosine residue or a methylated cytosine residue, and detecting a product generated due to said contacting, wherein the product is indicative of methylation of a cytosine residue in a CpG dinucleotide of the gene, thereby detecting methylation silencing of the gene of the test cell. Such a product can be detected using, for example, an electrophoresis method, a chromatography method, a mass spectrometry method, or a combination thereof.

[0015] In one aspect of such a method of detecting methylation of the 5' regulatory region of a target gene, the chemical reagent is hydrazine, thereby producing a hydrazine treated 5' regulatory region of the gene, wherein the hydrazine treated 5' regulatory region is further contacted with a reagent that cleaves hydrazine modified cytosine residues to

generate a product comprising fragments of the nucleic acid molecule comprising the gene; further separating the fragments according to molecular weight, and detecting a gap at a position known to contain a cytosine residue in the 5' regulatory region of the gene, wherein the gap is indicative of methylation of a cytosine residue in the CpG dinucleotide in the gene, thereby detecting methylation silencing of the gene of the test cell. The reagent used to cleave the hydrazine modified cytosine residue can be, for example, piperidine.

[0016] In another aspect of such a method of detecting methylation of the 5' regulatory region of a target gene, the chemical reagent can comprise bisulfite ions, whereby unmethylated cytosine residues in the 5' regulatory region of the gene are converted to bisulfite modified cytosine residues, wherein the bisulfite ion treated gene is further exposed to alkaline conditions, whereby bisulfite modified cytosine residues are converted to uracil residues, and detecting an amount or distribution of uracil residues in the 5' regulatory region of the bisulfite ion treated gene of the test cell, wherein a decrease in the amount or distribution of uracil residues in the 5' regulatory region of gene from the test cell, as compared to the amount or distribution of uracil residues in a corresponding bisulfite ion treated unmethylated gene following exposure to alkaline conditions, is indicative of methylation of cytosine residues in CpG dinucleotides in the 5' regulatory region of the gene.

[0017] In one aspect, the amount or distribution of uracil residues can be detected, for example, by determining the nucleotide sequence of the bisulfite modified 5' regulatory region of the gene following exposure to alkaline conditions. In another aspect, the amount or distribution of uracil residues also can be detected by contacting the bisulfite ion treated gene sequence, following exposure to alkaline conditions, with an oligonucleotide that selectively hybridizes to the 5' region regulatory of the gene containing uracil residues, and detecting selective hybridization of the oligonucleotide. Such a hybridizing oligonucleotide can further include a detectable label, for example, a radioisotope, a paramagnetic isotope, a luminescent compound, a chemiluminescent compound, a fluorescent compound, a metal chelate, an enzyme, a substrate for an enzyme, a receptor, or a ligand for a receptor, wherein selective hybridization of the oligonucleotide is detected by detecting the label. Alternatively, or in addition, the oligonucleotide is a substrate for a primer extension

reaction, wherein selective hybridization can be detected by detecting a product of the primer extension reaction.

[0018] In still another aspect, the amount or distribution of uracil residues can be detected by contacting the 5' regulatory region of the gene with an amplification primer pair (which includes a forward primer and a reverse primer) under conditions suitable for amplification, wherein at least one primer of the primer pair comprises an oligonucleotide that selectively hybridizes to a nucleotide sequence of the 5' regulatory region containing uracil residues, whereby generation of an amplification product is indicative of methylation of cytosine residues in CpG dinucleotides in the 5' regulatory region of the gene, thereby detecting methylation silencing of the gene of the test cell. Such a methylation specific amplification primer pair, which allows for methylation specific amplification of a sequence of a target gene, is exemplified by the primer pair set forth as SEQ ID NOS:1 and 2 (see, also, Table 4; SEQ ID NOS:65 to 127, including at least one forward primer (F1 or F2) and one reverse primer (R)).

[0019] In yet another aspect, the amount or distribution of uracil residues can be detected by contacting the 5' regulatory region of the gene with an amplification primer pair comprising a forward primer and a reverse primer under conditions suitable for amplification, wherein both primers of the primer pair selectively hybridize to a nucleotide sequence of the 5' regulatory region containing cytosine residues, but not to a corresponding nucleotide sequence of the 5' regulatory region containing uracil residues, whereby generation of an amplification product is indicative of a lack of methylation of cytosine residues in CpG dinucleotides in the 5' regulatory region of the gene, thereby detecting methylation silencing of the gene of the test cell. Such an unmethylation specific amplification primer pair, which allows for specific amplification of a sequence of a target gene comprising an unmethylated 5' regulatory region, is exemplified by the primer pair set forth as SEQ ID NOS:3 and 4.

[0020] In another aspect, the amount or distribution of uracil residues can be detected by contacting in the 5' regulatory region of the gene with a first amplification primer pair and a second amplification primer pair under conditions suitable for amplification, wherein the

first amplification primer pair comprises a forward primer and a reverse primer, wherein at least one primer of the first primer pair comprises an oligonucleotide that selectively hybridizes to a nucleotide sequence of the 5' regulatory region of the gene containing uracil residues, and wherein the second amplification primer pair comprises a forward primer and a reverse primer, wherein both primers of the second primer pair selectively hybridize to a nucleotide sequence of the 5' regulatory region of the gene containing cytosine residues, but not to a corresponding nucleotide sequence of the 5' regulatory region of the gene containing uracil residues, and wherein an amplification product, if any, generated by the first primer pair has a first length, and wherein an amplification product, if any, generated by the second primer pair has a second length, which is different from the first length, whereby the length of the amplification products is indicative of uracil residues and, therefore, methylation of cytosine residues in CpG dinucleotides in the 5' regulatory region of the gene.

[0021] In another embodiment, methylation silencing can be detected by contacting a test cell with a demethylating agent, and detecting reactivated expression of an RNA encoded by the gene as compared to a level of expression of the RNA in a corresponding test cell not contacted with a demethylating agent. The demethylating agent can be, for example, a methyltransferase inhibitor such as 5Aza-dC, and reactivated expression of an RNA encoded by an epigenetic silenced gene can be detected, for example, by detecting the expressed RNA, or a product thereof, for example, a reverse transcription-polymerase chain reaction (RT-PCR) product of the RNA.

[0022] The present methods of detecting epigenetically silenced genes conveniently can be adapted to a high throughput format, wherein the test cell, or extract of the test cell, is one of a plurality of test cells, or extracts of the test cells, or a combination thereof. The test cells, or extracts of the test cells, of the plurality can be the same or different, or a combination thereof, for example, duplicates, triplicates or more of a particular test cell sample, and a number of different test cell samples, which can, but need not, be arranged in an array, for example, an addressable array (e.g., on a solid support such as a microchip, a glass slide, or a bead). Furthermore, two or more genes can be examined for epigenetic silencing in a single sample of a test cell (or extract thereof) using, for example, differentially labeled

oligonucleotides, thus providing a multiplex format. In one embodiment, the test cells examined according to a high throughput and or multiplex assay can further include detecting methylation, if any, of cytosine residues in a CpG dinucleotide in a CpG island of the 5' regulatory region of the gene in a corresponding cell exhibiting regulated growth, or an extract of the corresponding cell.

[0023] The test cells (or extracts thereof) examined according to a method of the invention can comprise cells of a sample obtained from a subject, for example, a human subject. As such, the sample can be an organ sample, a tissue sample, or a cell sample, for example, an esophageal sample, a liver sample, a skin sample, a lymph node sample, a kidney sample, a lung sample, a muscle sample, a bone sample, a gastrointestinal tract sample, or a brain sample; or the sample can be a biological fluid, for example, a bone marrow, blood, serum, lymph, cerebrospinal fluid, saliva, sputum, stool, urine, or ejaculate, which contains nucleic acid molecules comprising one or more genes to be examined for epigenetic silencing.

[0024] The present invention also relates to a method of reducing or inhibiting unregulated growth of a cell exhibiting epigenetic silenced transcription of at least one gene associated with a cancer. Such a method can be practiced, for example, by restoring expression of a polypeptide encoded by the epigenetic silenced gene in the cell, thereby reducing or inhibiting unregulated growth of the cell. In one embodiment, expression of the polypeptide encoded by the epigenetic silenced gene can be restored by contacting the cell with a demethylating agent, a histone deacetylase inhibitor, or a combination thereof. In one aspect of this embodiment, at least one epigenetic silenced gene comprises a methylation silenced gene, and the cells are contacted with a demethylating agent such as 5Aza-dC. Generally, the cell is contacted with the demethylating agent by administering the demethylating agent to the subject such that it contacts the cells *in vivo*.

[0025] In another embodiment, expression of a polypeptide encoded by the epigenetic silenced gene in a cell is restored by introducing a polynucleotide encoding the polypeptide into the cell, whereby the polypeptide is expressed from the polynucleotide. The polynucleotide can, but need not, be contained in a vector, for example, a viral vector, and

can be formulated, for example, in a matrix such as a liposome, microbubbles, or the like. Generally, the polynucleotide is introduced into a cell by administering the polynucleotide to the subject such that the polynucleotide, which can be in a vector and/or formulated as above, contacts the cell *in vivo*.

[0026] A polynucleotide useful in such a method can be any polynucleotide corresponding to an epigenetically silenced gene. For example, where the cell is an ESCC cell, the epigenetic silenced gene can be a gene as set forth in Table 2, and the polynucleotide can be a nucleic acid molecule encoding a polypeptide encoded by the gene, such polynucleotides being available at the GenBank Accession No. as indicated in Table 2. For example, the epigenetic silenced gene can be an ApoD, NU, swisprosin-2, Hep27, KIF5C, keratin 14, transglutaminase 2, MUC1, IL-1 R2, crystallin alpha2, CLF-1, CRIP-1, Rad, HEM45, KLF6, follistatin related protein FLRG, XAP-5, Tbc1d1, cyclin G1 interacting protein, CRBP, metallothionein 1G, claudin-3, uncoupling protein-2, or apolipoprotein C1 gene, or a combination thereof. Where the cell is an HNSCC cell, the epigenetic silenced gene can be a gene as set forth in Table 5 or 6, and the polynucleotide can be a nucleic acid molecule encoding a polypeptide encoded by the gene, such polynucleotides being available at the GenBank Accession No. as indicated in Tables 5 and 6.

[0027] In one embodiment, the cell is an ESCC cell, and the epigenetic silenced gene comprises a methylation silenced gene, for example, a methylation silenced ApoD, NU, CLF-1, CRIP-1, claudin-3, uncoupling protein-2, metallothionein 1G, transglutaminase 2, or apolipoprotein C1 gene, or a combination thereof. In another embodiment, the cell is an ESCC cell, and the epigenetic silenced gene comprises a tumor suppressor gene, for example, an ApoD, NU, or CRIP-1 gene; or the tumor suppressor gene comprises a neuromedin B, or receptor of G protein signaling 2 (RGS2) gene; or the ESCC cell contains a combination of such tumor suppressor genes.

[0028] The present invention further relates to a method for treating a cancer patient, wherein cancer cells in the patient exhibit epigenetic silenced expression of at least one gene. Such a method can be performed, for example, by restoring expression of the at least

one epigenetic silenced gene in cancer cells in the subject, thereby treating the cancer patient. At least one epigenetic silenced gene can be a methylation silenced gene, and can, but need not, be a tumor suppressor gene or a gene that affects the activity or expression of a tumor suppressor gene.

[0029] In one embodiment, cancer cells of the cancer patient contain at least one methylation silenced gene, and the method comprises administering a demethylating agent to the subject in an amount sufficient to restore expression of the methylation silenced gene in cancer cells in the subject. In another embodiment, cancer cells of the cancer patient contain at least one epigenetic silenced gene, and the method comprises administering at least one polynucleotide encoding a polypeptide encoded by an epigenetic silenced gene to the subject under conditions sufficient for expression of the at least one polypeptide in cancer cells in the subject. The polynucleotide can be contained in a vector such as a viral vector; and/or can be formulated with a matrix such as liposomes or microbubbles.

[0030] A cancer treated according to a method of the invention can be any cancer comprising cancer cells containing at least one epigenetic silenced gene associated with the cancer, including, for example, a carcinoma or a sarcoma. In one embodiment, the cancer is an esophageal squamous cell carcinoma, and the epigenetic silenced gene includes one or more genes as set forth in Table 2. For example, the epigenetic silenced gene can be an ApoD, NU, swisprosin-2, Hep27, KIF5C, keratin 14, transglutaminase 2, MUC1, IL-1 R2, crystallin alpha2, CLF-1, CRIP-1, Rad, HEM45, KLF6, follistatin related protein FLRG, XAP-5, Tbc1d1, cyclin G1 interacting protein, CRBP, metallothionein 1G, claudin-3, uncoupling protein-2, or apolipoprotein C1 gene, or a combination thereof. In one aspect, the epigenetic silenced gene(s) include at least one methylation silenced gene, for example, an ApoD, NU, CLF-1, CRIP-1, claudin-3, uncoupling protein-2, metallothionein 1G, transglutaminase 2, or apolipoprotein C1 gene, or a combination thereof. In another aspect, the epigenetic silenced gene(s) include at least one tumor suppressor gene, for example, an ApoD, NU, and/or CRIP-1 gene; or a neuromedin B and/or RGS2 gene; or a combination thereof. In another embodiment, the cancer is a head and neck cancer and the epigenetic silenced gene includes one or more genes as set forth in Tables 5 and 6.

[0031] The present invention also relates to a method for selecting a therapeutic strategy for treating a cancer patient. Such a method can be performed, for example, by identifying at least one epigenetically silenced gene associated with the cancer according a genome screening method of the invention as disclosed herein; and selecting an agent useful for restoring expression of the at least one epigenetically silenced gene in cancer cells of the patient. The agent can be, for example, a polynucleotide encoding a polypeptide otherwise expressed from the epigenetically silenced gene(s), for example, a polynucleotide encoding a polypeptide encoded by a gene listed in Table 2 such as an ApoD, NU, swisprosin-2, Hep27, KIF5C, keratin 14, transglutaminase 2, MUC1, IL-1 R2, crystallin alpha2, CLF-1, CRIP-1, Rad, HEM45, KLF6, follistatin related protein FLRG, XAP-5, Tbc1d1, cyclin G1 interacting protein, CRBP, metallothionein 1G, claudin-3, uncoupling protein-2, or apolipoprotein C1 gene, or a combination thereof.

[0032] In one embodiment, the identified epigenetic silenced gene comprises at least one methylation silenced gene, and the agent selected is one useful for restoring expression of the at least one methylation silenced gene in the cancer cells. In one aspect of this method, the selected agent comprises a polynucleotide encoding a polypeptide otherwise encoded by the methylation silenced gene(s), for example, an ApoD, NU, CLF-1, CRIP-1, claudin-3, uncoupling protein-2, metallothionein 1G, transglutaminase 2, or apolipoprotein C1 gene. In another aspect of this method, the selected agent comprises a demethylating agent, for example, 5Aza-dC.

[0033] In another embodiment, the identified epigenetic silenced gene comprises at least one tumor suppressor gene, and the agent selected is one useful for restoring a polypeptide encoded by the epigenetic silenced tumor suppressor gene in the cancer cells. For example, the tumor suppressor gene can be an ApoD, NU, or CRIP-1 gene, or a neuromedin B or RGS2 gene, or a combination of such genes, and the selected agent can be a polynucleotide encoding an ApoD, NU, CRIP-1, neuromedin B, and/or RGS2 gene product.

[0034] The present invention also relates to a method of treating a subject suffering from an ESCC, wherein cells associated with the ESCC contain at least one epigenetic silenced gene. Such a method can be performed, for example, by administering an amount of an

agent that restores expression of the at least one epigenetic silenced gene to the subject sufficient to restore expression of the epigenetic silenced gene in cells associated with the ESCC, thereby treating the subject. In one embodiment, the agent comprises a polynucleotide encoding the at least one epigenetically silenced gene, particularly a polynucleotide comprising a coding sequence of a gene as listed in Table 2, for example, a polynucleotide comprising a coding sequence of an ApoD, NU, swisprosin-2, Hep27, KIF5C, keratin 14, transglutaminase 2, MUC1, IL-1 R2, crystallin alpha2, CLF-1, CRIP-1, Rad, HEM45, KLF6, follistatin related protein FLRG, XAP-5, Tbc1d1, cyclin G1 interacting protein, CRBP, metallothionein 1G, claudin-3, uncoupling protein-2, or apolipoprotein C1 gene, or a combination thereof.

[0035] In another embodiment, at least one epigenetic silenced gene is a methylation silenced gene, and the agent for treating the subject comprises a polynucleotide encoding a polypeptide encoded by the methylation silenced gene. For the polynucleotide can comprises a coding sequence of an ApoD, NU, CLF-1, CRIP-1, claudin-3, uncoupling protein-2, metallothionein 1G, transglutaminase 2, or apolipoprotein C1 gene, or a combination thereof.

[0036] In still another embodiment, at least one epigenetically silenced gene comprises at least one tumor suppressor gene, and the method of treating a subject comprises restoring expression of the tumor suppressor gene in ESCC cells of the subject. For example, the tumor suppressor gene can be an ApoD, NU, or CRIP-1; or can be a neuromedin B, or RGS2 gene; or can be a combination including at least one of such genes; and the agent can be a polynucleotide encoding one or more of the epigenetically silenced tumor suppressor genes. An agent useful in a method of the invention can be administered to a subject directly to the site of the cells, or locally or systemically such that it can contact the ESCC cells in the subject.

[0037] The present invention further relates to an isolated oligonucleotide, which has a nucleotide sequence as set forth in any one of SEQ ID NOS:1 to 127, as well as to a plurality of isolated oligonucleotides, which includes at least two of the isolated oligonucleotides as set forth in SEQ ID NOS:1 to 127. In addition, the invention relates to

an amplification primer pair, which includes a forward primer and a reverse primer as exemplified by SEQ ID NOS:1 and 2; and SEQ ID NOS:3 and 4 (see, also, Table 3, SEQ ID NOS:7 and 8, SEQ ID NOS:9 and 10, etc.; and Table 4, SEQ ID NOS:65 and 67 or SEQ ID NOS:66 and 67; SEQ ID NOS:68 and 69; SEQ ID NOS:70 and 72 or SEQ ID NOS:71 and 72, etc.), which can amplify a nucleotide sequence of a gene as listed in Table 2. In one aspect, an amplification primer pair of the invention can be used to specifically amplify a methylated 5' regulatory region of the nucleic acid molecule, such amplification primer pairs being exemplified by SEQ ID NOS:1 and 2, which can amplify an ApoD gene having a methylated 5' regulatory region, and by the primer pairs comprising a forward primer (F1 or F2) and a reverse primer (R) as set forth in Table 4; SEQ ID NOS:65 to 124), which can amplify a gene as indicated having a methylated 5' regulatory region. In another aspect, an amplification primer pair of the invention can be used to specifically amplify an unmethylated 5' regulatory region of the nucleic acid molecule, such amplification primer pairs being exemplified by SEQ ID NOS:3 and 4, which can amplify an ApoD gene having an unmethylated 5' regulatory region.

[0038] The present invention also relates to a kit, which contains at least one isolated oligonucleotide of the invention, including, for example, a plurality of such isolated oligonucleotides. In one embodiment, a plurality of isolated oligonucleotides of a kit of the invention includes at least one amplification primer pair (i.e., a forward primer and a reverse primer), and can include a plurality of amplification primer pairs, including. As such, a kit of the invention can contain, for example, one or a plurality of methylation specific amplification primer pairs, unmethylation specific amplification primer pairs, or a combination of methylation specific amplification primer pairs and unmethylation specific amplification primer pair, including methylation specific primer pairs and unmethylation specific primer pairs useful for amplifying a methylated form or an unmethylated form of a particular gene that is known to be or suspected of being methylation silenced in one or more types of cancer cells.

[0039] A kit of the invention can further include additional reagents, which can be useful, for example, for a purpose for which the oligonucleotides of the kit are useful. For example, where a kit contains one or a plurality of methylation specific and/or

unmethylation specific amplification primers, the kit can further contain, for example, control polynucleotides, which can be methylated or unmethylated; one or more reagents that modify methylated cytosine residues, and/or one or more reagents for performing an amplification reaction. Where the kit contains one or plurality of oligonucleotides that selectively hybridize to a methylated or to an unmethylated gene sequence, the kit can further contain, for example, a methylation sensitive restriction endonuclease.

### BRIEF DESCRIPTION OF THE DRAWINGS

[0040] Figure 1 provides a flowchart for selecting candidate tumor suppressor genes (TSGs). Three esophageal squamous cell carcinoma (ESCC) cell lines were screened for candidate TSGs following treatment with 1 to 5  $\mu$ M 5-aza-2'-deoxycytidine (5Aza-dC)  $\pm$  300 nM trichostatin A (TSA) by cRNA hybridization to a 12,599 oligonucleotide microarray. More than 500 unique genes that showed  $\geq 3$  fold increase after treatments were identified (see Table 1). The number of candidate genes was diminished by selecting genes that were commonly up-regulated in the ESCC cell lines examined (120 genes), and further removed several genes by expression profiling and elimination of unknown genes. Of the 58 remaining genes, 22 were selected and examined for promoter hypermethylation by direct sequencing or methylation specific PCR (MSP) in ESCC cell lines, and 13 were confirmed. Ten of these 13 genes were methylated in ESCC tissues; and 3 of the 10 were examined and found to possess growth suppression activity in a colony focus assay. Superscript letters indicate as follows: a) re-expressed in more than two ESCC cell lines; b) excluded if no evidence of expression in normal esophagus; c) 22 genes with CpG rich promoter selected to test for methylation in cell lines; d) 10/13 genes confirmed to harbor promoter methylation in primary tumor tissues; and e) 3/10 genes selected and confirmed to possess tumor suppressive activity.

[0041] Figure 2 demonstrates the methylation and expression in primary tumors. Methylation status was examined in primary tumors for 13 tested genes that showed methylation in ESCC cell lines. Shaded boxes indicate methylation of the promoter region; Speckled box indicates partial (low-level) methylation by direct sequencing. T, Tumor tissues; N, normal esophageal mucosa.

[0042] Figure 3 shows the results of a colony formation assay in KYSE30 ESCC cells. Results are an average of 3 independent experiments. Colony formation efficiency of cells transfected with Stat3C (2), Apo D (3), CRIP1 (4), or p53 (5), as compared to mock (1; empty pcDNA3<sup>TM</sup> vector). Mock colony number was arbitrarily set at 100% colony formation; Apo D = 61%; CRIP1 = 18% for CRIP1. In other experiments, colony formation after 2 weeks of selection with G418 of pcDNA3 (mock) transfected cells with PBS or neuromedin U (final concentration 100  $\mu$ M) revealed that colony formation efficiency was reduced to 43% in the neuromedin U treated cells.

#### DETAILED DESCRIPTION OF THE INVENTION

[0043] The present invention is based on the development of a method for identifying epigenetically silenced genes, particularly tumor suppressor gene, in the genome of a cell exhibiting, or predisposed to suspected of exhibiting unregulated growth, for example, a cancer cell genome. The method is exemplified by the identification of 565 genes that were up-regulated in esophageal squamous cell carcinoma (ESCC) cells following treatment with a demethylating agent, a histone deacetylase inhibitor, or both, , including methylation silenced genes and tumor suppressor genes (see Table 1). Further, fifty-eight commonly up-regulated genes were identified, 53 of which contained CpG islands, including 44 genes containing dense CpG islands. Twenty-five of the 53 genes were randomly selected and found to be exhibit robust re-expression following treatment with a demethylating agent, and three of the genes were confirmed to have tumor suppressor activity (see Example 1). In addition, the genomic screening method is further exemplified by the identification of genes that are epigenetically silenced in head and neck squamous cell carcinoma (HNSCC) cells (see Tables 5 and 6, and Example 2). Accordingly, the present invention provides a method for identifying epigenetically silenced genes associated with a cancer, and further provides methods of detecting a cancer associated with epigenetic silencing of gene expression, methods of treating a patient having such a cancer, and compositions useful for practicing such methods.

[0044] Promoter hypermethylation is a common pathway for tumor suppressor gene inactivation. As disclosed herein, a method is provided for identifying methylated genes based on pharmacological unmasking of epigenetic silencing in esophageal cancer cell

lines. This approach and selection algorithm is robust in identifying a number of novel methylated genes in primary esophageal tumor tissues. The identified methylated genes provide diagnostic and therapeutic targets, and further provide insight into tumor biology. Three of the identified genes, neuromedin U (NU), cystein rich intestinal protein 1 (CRIP1), and apolipoprotein D (ApoD) demonstrated potent tumor suppressive activity when overexpressed in carcinoma cells.

[0045] A comprehensive survey of commonly inactivated tumor suppressor genes in ESCC cells was performed based on functional reactivation of epigenetically silenced tumor suppressor genes by 5-aza-2'-deoxycytidine (5Aza-dC) and trichostatin A (TSA) using microarrays containing 12,599 genes. Among 58 genes identified by this approach, 44 (76%) harbored dense CpG islands in the promoter regions. Thirteen of twenty-two tested gene promoters were methylated in cell lines, and 10 in primary ESCC cells, accompanied by silencing at the mRNA level. Potent growth suppressive activity of 3 genes including CRIP1, ApoD, and NU in ESCC cells was demonstrated by colony focus assays. The results disclosed herein demonstrate that pharmacologic reversal of epigenetic silencing is a powerful approach for comprehensive identification of tumor suppressor genes in human cancers.

[0046] Cancer of the esophagus is the eighth most common malignancy and ranks as the sixth most frequent cause of death worldwide (Pissani et al., *Int. J. Cancer* 80:870-873, 1999). The frequency of different histologic types of esophageal carcinoma varies, but throughout the world squamous cell carcinoma is the predominant type. Considerable epidemiological evidence suggests that alcohol, tobacco, diets deficient in vitamins/protective antioxidants, carcinogens (e.g., frequent consumption of pickled vegetables) and thermal injuries are important in the pathogenesis of ESCC (see, for example, Chen et al., *Int. J. Cancer* 820-822, 1995; Garidou et al., *Int. J. Cancer* 68:295-299, 1996). Recent advances in molecular biology have revealed common genetic and/or epigenetic alterations of the p53 and p16/Rb tumor suppressor pathways in human ESCC (Xu et al., *Cancer Res.* 62, 3493-3497, 2002; Montesano et al., *Int. J. Cancer* 69:225-235, 1996; Mandard et al., *Mutat. Res.* 462:335-342, 2000). Further identification of molecular targets would enable the prevention, diagnosis, and treatment of ESCC to be approached at

the molecular level. However, as in other cancers, a genome-wide comprehensive survey of commonly inactivated tumor suppressor genes (TSGs) in ESCC has remained elusive.

[0047] In addition to genetic alterations, alterations in DNA methylation, an epigenetic process present in mammalian cells, are also a hallmark of human cancer (Baylin et al., *Hum. Mol. Genet.* 10:687-692, 2001). The promoter regions of many genes, particularly "housekeeping" genes, are populated by many CpG dinucleotides, which are often underrepresented in the remainder of the genome. These regions have been termed "CpG islands", and, with the exception of genes on the inactive X chromosome and imprinted genes, CpG islands are protected from methylation in normal cells (see Baylin et al., *supra*, 2001). This protection is critical, since methylation of CpG islands is associated with loss of expression of that particular gene. In carcinogenesis, global hypermethylation is often accompanied by dense hypermethylation of specific promoters (Yoshikawa et al., *Nature Genet.* 28:29-35, 2001, which is incorporated herein by reference; see, also, Merlo et al., *Nature Med.* 1:686, 1995; Dammann et al., *Nat. Genet.* 25:315-319, 2000; Li et al., *Cell* 109:113-124, 2002). Many studies have demonstrated that the silencing of tumor suppressor genes associated with promoter hypermethylation is a common feature in human cancer, and serves as an alternative mechanism for loss of tumor suppressor gene function. For example, *p16* hypermethylation was associated with loss of expression and was a common feature of many solid tumor malignancies (Merlo et al., *supra*, 1995). Hypermethylation also was associated with inactivation of the tumor suppressor gene, *VHL*, and occurred in a subset of clear cell renal cancers without inactivating point mutations (Herman et al., *Proc. Natl. Acad. Sci. USA* 91 :9700-9704, 1994; Meyer et al., *Int. J. Cancer* 5:650-653, 2000), while hypermethylation associated loss of *p15* expression was a feature of many acute leukemias (Herman et al., *Cancer Res.* 56:722-727, 1996). The transcriptional silencing of other tumor suppressor genes such as the mismatch repair gene has established hypermethylation as a common mechanism for loss of tumor suppressor function in human cancer. Thus, an increasing number of tumor suppressor genes display both genetic and epigenetic inactivation in human tumors.

[0048] Because promoter hypermethylation is linked to silencing of gene expression, knowledge of methylation patterns across the genome, sometimes dubbed "the methylome"

(Feinberg, *Nat. Genet.* 27:9-10, 2001), can provide a means to potentially identify TSGs inactivated during tumor formation. 5Aza-dC, which is incorporated into genomic DNA and forms a covalent complex with methyltransferase active sites, has been used to unravel epigenetic inactivation. This suicide inhibition depletes methyltransferase activity resulting in generalized demethylation. However, chromatin is a complex of DNA and histones, and histone acetylation also impairs gene transcription (Pennisi, *Science* 275:155-157, 1997; Marks et al., *Nat. Rev. Cancer* 3:194-202 2001). In addition, DNA methylation helps model histone acetylation (Gray and Teh, *Curr. Mol. Med.* 1:401-429, 2001). Methyl-CpG-binding protein MeCP2 appears to reside in a complex with histone deacetylase activity, while DNA methyltransferase binds histone deacetylase 2 (HDAC2) and a co-repressor, DMAP1 (Rountree et al., *Nat. Genet.* 25:269-277, 2000). Thus, densely methylated DNA associates with transcriptionally repressive chromatin characterized by the presence of underacetylated histones. TSA, a histone deacetylase inhibitor, reverses formation of transcriptionally repressive chromatin on methylated promoter templates (Yoshida and Horinouchi, *Ann. NY Acad. Sci.* 886:23-36, 1999). Epigenetic alterations are thus dynamically linked, and synergy between demethylation and histone deacetylase inhibition using TSA reactivated genes silenced in carcinoma more robustly than 5Aza-dC alone (Cameron et al., *Nature Genet.* 21:103-107, 1999; Suzuki et al., *Nature Genet.* 31:141-149, 2002). As disclosed herein, pharmacological unmasking of ESCC cells with 5Aza-dC and TSA, followed by cRNA microarray analysis, comprehensively identified epigenetically inactivated genes in cancer. This approach identified a large number of genes with dense promoter hypermethylation, and further examination revealed that a subset of the identified genes was frequently inactivated in primary tumors and displayed tumor suppressor activity.

[0049] Accordingly, methods are provided for identifying epigenetically silenced genes, for example, methylation silenced genes, including tumor suppressor genes that are associated with a cancer. In one embodiment, the invention provides a method of identifying at least one epigenetically silenced gene associated with at least one cancer. As used herein, the term "at least one" means 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more. For example, the disclosed microarray method identified 565 genes that were epigenetically silenced, as determined by their ability to be up-regulated following treatment with a demethylating agent and/or histone deacetylase inhibitor in ESCC cells. Furthermore, it was determined

that several of the genes that were identified as epigenetically silenced in ESCC had the characteristics expected of TSGs (see, also, Tables 2, 5 and 6).

[0050] The term "epigenetically silenced", when used in reference to a gene, means that the gene is not being transcribed, or is being transcribed at a level that is decreased with respect to the level of transcription of the gene in a corresponding control cell (e.g., a normal cell), due to a mechanism other than a genetic change. Epigenetic mechanisms of gene silencing are well known and include, for example, hypermethylation of CpG dinucleotides in a CpG island of the 5' regulatory region of a gene, and structural changes in chromatin due, for example, to histone acetylation, such that gene transcription is reduced or inhibited. Methods for detecting epigenetic silencing of a gene are disclosed herein and include, for example, detecting re-expression (reactivation) of the gene following contact of a cell with an agent that relieves the epigenetic silencing, for example, with a demethylating agent where the silencing is due to hypermethylation.

[0051] As used herein, the term "methylation" or "hypermethylation", when used in reference to a gene, means that cytosine residues of CpG dinucleotides in a CpG island associated with the gene are methylated at the 5'-position, i.e., 5'-methylcytosine. The term "methylation status" is used herein to refer to a relative abundance, including the presence or absence, of methylated cytosine residues of CpG dinucleotides in a CpG island. In general, the cytosine residues in a CpG island are not methylated in a transcriptionally active gene and, therefore, the detection of methylated cytosine residues in a CpG island indicates that expression of the gene is reduced or inhibited. Accordingly, reference herein to a "methylation silenced" gene means that the gene is not being transcribed, or is being transcribed at a level that is decreased with respect to the level of transcription of the gene in a corresponding control cell (generally a normal cell) due to hypermethylation of CpG dinucleotides in a CpG island of the 5' regulatory region of the gene. A consequence of methylation silenced gene expression is that a cell containing the gene has reduced levels of, or completely lacks, a polypeptide encoded by the gene (i.e., the gene product) such that any function normally attributed to the gene product in the cell is reduced or absent.

[0052] The present invention relates to a method of identifying at least one epigenetically silenced gene associated with a cancer. Such a method can be performed, for example, by contacting an array of nucleotide sequences representative of a genome with nucleic acid molecules corresponding to RNA expressed in cancer cells contacted with at least one agent that reactivates expression of epigenetically silenced genes, under conditions suitable for selective hybridization of nucleic acid molecules to complementary nucleotide sequences of the array; and detecting increased hybridization of nucleic acid molecules of the cancer cells contacted with the agent(s) to a subpopulation of nucleotide sequences of the array as compared to a level of hybridization, if any, of nucleic acid molecules corresponding to RNA expressed in the cancer cells to at least one nucleotide sequence of the subpopulation of nucleotide sequences, under said conditions, whereby increased selective hybridization identifies reactivated expression of an epigenetically silenced gene.

[0053] As used herein, the term "array of nucleotide sequences representative of a genome" means an organized group of nucleotide sequences that are linked to a solid support, for example, a microchip or a glass slide, wherein the sequences can hybridize specifically and selectively to nucleic acid molecules expressed in a cell. The array is selected based on the organism from which the cells to be examined are derived, and, therefore, generally is representative of the genome of a eukaryotic cell, particularly a mammalian cell, and preferably a human cell. In general, an array of probes that is "representative" of a genome will identify at least about 10% of the expressed nucleic acid molecules in a cell, generally at least about 20% or 40%, usually about 50% to 70%, particularly at least about 80% or 90%, and preferably will identify all of the expressed nucleic acid molecules. It should be recognized that the greater the representation, the more likely all genes that are epigenetically silenced in a cancer will be identified. Arrays containing nucleotide sequences representative of specified genomes can be prepared using well known methods, or obtained from a commercial source (e.g., Affymetrix; Invitrogen Corp.), as exemplified by the GeneChip<sup>TM</sup> Human Genome U95AV2 array (Affymetrix) used in the present studies (see Example 1).

[0054] Reference herein to "nucleic acid molecules corresponding to RNA" of a cell means RNA such as mRNA or polyA<sup>+</sup> RNA, cDNA generated using RNA from the cell as

a template, or cRNA generated using RNA or cDNA as a template. For practicing a method of the invention, the nucleic acid molecules corresponding to RNA of a cell generally are detectably labeled, for example, with a radioisotope, a paramagnetic isotope, a luminescent compound, a chemiluminescent compound, a fluorescent compound, a metal chelate, an enzyme, a substrate for an enzyme, a receptor, or a ligand for a receptor; or are capable of being detected, for example, using a detectably labeled probe, such that hybridization of the nucleic acid molecules to nucleotide sequences of the array can be detected. Thus, the nucleic acid molecules corresponding to RNA that are contacted with the nucleotide sequences of the array can be DNA or RNA, including, for example, cDNA, cRNA, mRNA, or any other nucleic acid molecules representative of RNA expressed in a cell. The agent that reactivates expression of epigenetically silenced genes can be a demethylating agent such as a methyltransferase inhibitor (e.g., 5Aza-dC), a histone deacetylase inhibitor (e.g., TSA), or a combination thereof.

[0055] According to a method of the invention, at least one (e.g., 1, 2, 3, 4, 5, or more) epigenetically silenced gene can be associated with at least one (e.g. 1, 2, 3, or more) cancer. The cancer can be, for example, a carcinoma or a sarcoma, including one or more specific types of cancer, e.g., an alimentary/gastrointestinal tract cancer, a liver cancer, a skin cancer, a breast cancer, an ovarian cancer, a prostate cancer, a lymphoma, a leukemia, a kidney cancer, a lung cancer, a muscle cancer, a bone cancer, or a brain cancer. Epigenetically silenced genes associated with a cancer are exemplified herein by the genes listed in Table 2 (and for which GenBank Accession numbers are provided), which are associated with ESCC. With reference to Table 2, epigenetically silenced genes in ESCC cells that can be reactivated due to contact of the cells with a demethylating agent, a histone deacetylase inhibitor, or a combination thereof, include an apolipoprotein D (ApoD) gene, neuromedin U (NU) gene, swisprosin-2 gene, Hep27 gene, KIF5C gene, keratin 14 gene, transglutaminase 2 gene, MUC1 gene, interleukin-1 receptor 2 (IL-1 R2) gene, crystallin alpha2 gene, cysteine-rich protein with LIM (CRIP-1) gene, Rad gene, HEM45 gene, KLF6 gene, follistatin related protein FLRG gene, XAP-5 gene, Tbc1d1 gene, cyclin G1 interacting protein gene, or a combination thereof.

[0056] In one embodiment, the epigenetically silenced genes include one or more of an ApoD gene, NU gene, swisprosin-2 gene, cytokine-like factor-1 (CLF-1) gene, CRIP-1 gene, cellular retinol binding protein (CRBP) gene, metallothionein 1G gene, keratin 14 gene, IL-1 R2 gene, or crystallin alpha2 gene. In another embodiment, at least one epigenetically silenced gene is a methylation silenced gene, for example, an ApoD, NU, CLF-1, CRIP-1, claudin-3, uncoupling protein-2, metallothionein 1G, transglutaminase 2, or apolipoprotein C1 gene, or a combination thereof. In still another embodiment, the epigenetically silenced gene is a tumor suppressor gene, for example, an ApoD gene, a NU gene, or a CRIP-1 gene, each of which, as disclosed herein, exhibits tumor suppressor activity; and/or a neuromedin B gene and/or a receptor of G protein signaling 2 (RGS2) gene.

[0057] The silencing of gene transcription associated with aberrant DNA methylation of CpG dinucleotides in normally unmethylated gene promoter regions is the most widely studied epigenetic abnormality in tumorigenesis. The binding of protein complexes consisting of methyl-CpG-binding domains, transcriptional co-repressors, chromatin remodeling proteins and histone deacetylases to hypermethylated DNA regions results in a transcriptionally repressed (silenced) chromatin state. In eukaryotic cells, methylation of cytosine residues that are immediately 5' to a guanosine residue occurs predominantly in CG poor regions. In contrast, CpG islands generally remain unmethylated in normal cells, except during X chromosome inactivation and parental specific imprinting, where methylation of 5' regulatory regions is associated with transcriptional repression. *De novo* methylation of the retinoblastoma (Rb) gene has been demonstrated in a small fraction of retinoblastomas (Sakai et al., *Am. J. Hum. Genet.* 48:880, 1991), and aberrant methylation of the VHL gene was found in a subset of sporadic renal cell carcinomas (Herman et al., *Proc. Natl. Acad. Sci. USA* 91:9700-9704, 1994). Expression of a tumor suppressor gene can also be abolished by *de novo* DNA methylation of a normally unmethylated 5' CpG island (see, for example, Issa et al., *Nature Genet.* 7:536, 1994; Merlo et al., *Nature Med.* 1:686, 1995; Herman et al., *Cancer Res.* 56:722, 1996).

[0058] Aberrant methylation of promoter regions in CpG islands also has been associated with the development of cancer. In hematopoietic malignancies, for example,

hypermethylation of E-cadherin (Graff et al., *Cancer Res.* 55:5195-5199, 1995), DAP-kinase (Katzenellenbogen et al., *Blood* 93:4347-4353, 1999), and the cell cycle regulators p15<sup>INK4B</sup> and p16<sup>INK4A</sup>, is associated with gene inactivation (Herman et al., *Cancer Res.* 57:837-841 1997; Melki et al., *Blood* 95:3208-3213, 2000; Ng et al., *Clin. Canc. Res.* 7:1724-1729, 2001). Transcriptional silencing due to hypermethylation also has been detected in the CDKN2A gene (Herman et al., *Cancer Res.* 55:4525-4530, 1995), MGMT (Esteller et al., *Cancer Res.* 59:793-797, 1999), and MLH1 gene (Herman et al., *Proc. Natl. Acad. Sci. USA* 95:6870-6875, 1998).

[0059] Hypermethylation of a CpG island at chromosome position 17p13.3 has been observed in multiple common types of human cancers (Makos et al., *Proc. Natl. Acad. Sci. USA* 89:1929, 1992; Makos et al., *Cancer Res.* 53:2715, 1993; Makos et al., *Cancer Res.* 53:2719, 1993), and coincides with timing and frequency of 17p loss and p53 mutations in brain, colon, and renal cancers. Silenced gene transcription associated with hypermethylation of the normally unmethylated promoter region CpG islands has been implicated as an alternative mechanism to mutations of coding regions for inactivation of tumor suppressor genes (Baylin et al., *Cancer Cells* 3:383, 1991; Jones and Buckley, *Adv. Cancer Res.* 54:1-23, 1990). This change also has been associated with the loss of expression of VHL, a renal cancer tumor suppressor gene on 3p (Herman et al., *supra*, 1994), the estrogen receptor gene on 6q (Ottaviano et al., *Cancer Res.* 54:2552, 1994), and the H19 gene on 11p (Steenman et al., *Nature Genetics*, 7:433, 1994).

[0060] The present invention also relates to a method for identifying a cell that exhibits or is predisposed to exhibiting unregulated growth. Such a method can be performed, for example, by detecting, in a test cell, epigenetic silencing of at least one gene as set forth in Table 2, or a combination of such genes. In one embodiment, a method of the invention requires, in part, a comparison of the methylation status of a gene in a test cell or sample with the methylation status of a corresponding gene in a corresponding cell exhibiting regulated growth. As used herein, the term "corresponding" means a reference material, with which a test material is being compared. Generally, the reference material provides a control or standard with which the test material is compared. For example, reference to a corresponding unmethylated ApoD gene, with respect to an ApoD gene being examined for

methylation status, means that the unmethylated ApoD gene is the same type of gene as the ApoD gene being examined for methylation status, e.g., the test gene and the corresponding unmethylated gene are both human ApoD genes. Reference to a corresponding cell exhibiting regulated growth, with respect to a test cell, generally refers to a normal cell, i.e., a cell that has a cell cycle and growth pattern characteristic of a population of such cells in a healthy individual, for example, a normal esophageal epithelial cell where the test cell being examined is suspected of being an ESCC cell.

[0061] A method of the invention is practiced using a sample comprising a test cell, or an extract of the test cell that includes nucleic acid molecules of the cell, particularly genomic DNA, including all or a portion comprising the CpG island of a 5' regulatory region of the gene that is to be examined for methylation status. Generally, the test cell is a cell that is suspected of being a cell that exhibits unregulated growth, for example, a biopsy sample of suspicious lesion, or is a cell that is (or was) in proximity to a premalignant or malignant cell, for example, cell samples taken at one or few places outside of the region of a suspicious lesion, such test cell providing an indication, for example, of the extent to which a surgical procedure should be performed, or a cell sample taken from a surgical margin, such test cells being useful for determining whether a cancer has been completely removed, or for determining whether a cancer has recurred.

[0062] A test cell examined according to a method of the invention also can be a primary cell that has been obtained from a subject and placed in culture, for example, for the purpose of establishing a primary cell culture that exhibits substantially the same growth characteristics as the cells from which the culture was established, or for the purpose of treating and/or expanding the cells for readministration to the subject. For example, esophageal epithelial cells can be obtained from a cancer patient suffering from a ESCC, wherein the cells exhibit methylation silenced expression of one or more genes associated with the cancer. The cells can be treated in culture using one or more agent to be tested for an ability to restore expression of the silenced gene(s), thus providing a means to identify an agent that can be useful for treating the cancer patient, or another patient having a ESCC characterized by methylation silencing of one or more of the same genes.

[0063] A test cell can be obtained from a subject in any way typically used in clinical setting for obtaining a sample containing the cells. For example, the test cells (or a sample comprising the test cells) can be obtained by a biopsy procedure such as needle biopsy of an organ or tissue containing the cells to be tested. As such, the test cells can be obtained from an alimentary tract sample, gastrointestinal tract sample, a liver sample, a bone marrow sample, a skin sample, a lymph node sample, a kidney sample, a lung sample, a muscle sample, a bone sample, a brain sample, or the like. The test cell also can be a component of a biological fluid, for example, blood, lymph, cerebrospinal fluid, saliva, sputum, stool, urine, or ejaculate. If appropriate, the test cells also can be obtained by lavage, for example, for obtaining test cells from the colon, uterus, abdominal cavity, or the like, or using an aspiration procedure, for example, for obtaining a bone marrow sample.

[0064] A method of the invention also can be practiced using an extract of a test cell, wherein the extract includes nucleic acid molecules of the test cell, particularly genomic DNA, including all or a CpG island containing portion of the gene or genes to be examined. The extract can be a crude extract comprising, for example, a freeze-thawed sample of a tissue containing the test cells; can comprise partially purified genomic DNA, which can include, for example, components of the nuclear matrix; or can comprise substantially purified genomic DNA, which is obtained, for example, following treatment with a protease and alcohol precipitation. In certain embodiments, the test cell also can be a component of a histologic sample that is embedded in paraffin.

[0065] Where the epigenetic silencing includes methylation silencing, the method for identifying a cell that exhibits or is predisposed to exhibiting unregulated growth is performed by detecting methylation of one or more target genes in the cell. Methylation of a CpG dinucleotide in a CpG island of a gene can be detected using any of various well known methods for detecting CpG methylation of a nucleic acid molecule. Such methods include contacting the gene with one or a series of chemical reagents that selectively modify either unmethylated cytosine residues or methylated cytosine residues, but not both, such that the presence or absence of the modification can be detected; contacting the gene sequence with a methylation sensitive restriction endonuclease, which has a recognition site that includes a CpG dinucleotide, and that cleaves a recognition site either having a

methyated cytosine residue of the CpG or lacking a methyated cytosine residue of the CpG, but not both, such that the presence or absence of cleavage of the sequence can be detected; or contacting a nucleic acid molecule comprising the gene with an oligonucleotide probe, primer, or amplification primer pair that selectively hybridizes to the gene sequence and allows a determination to be made as to whether the CpG methylation is present. Examples of such methods are provided herein, and modifications and variations on such methods are well known in the art.

[0066] Methylation of a target gene can be detected, for example, by contacting a region comprising a 5' regulatory region of a nucleic acid molecule comprising the gene with a methylation sensitive restriction endonuclease, which cleaves a recognition site in the 5' regulatory region comprising a methyated cytosine residue of a CpG dinucleotide, whereby cleavage of the nucleic acid molecule is indicative of methylation and, therefore, methylation silencing of the gene of the test cell. Methylation sensitive restriction endonucleases are well known and include, for example, Acc III, Ban I, BstNI, Msp I, and Xma I. Alternatively, or in addition, methylation silencing can be detected by contacting a region comprising a 5' regulatory region of a nucleic acid molecule comprising the gene with a methylation sensitive restriction endonuclease, which cleaves a recognition site in the 5' regulatory region comprising a methyated cytosine residue of a CpG dinucleotide, provided the cytosine residue of the CpG dinucleotide is unmethylated, whereby a lack of cleavage of the nucleic acid molecule is indicative of methylation silencing of the gene of the test cell. Such methylation sensitive restriction endonucleases are exemplified by Acc II, Ava I, BssH II, BstU I, Hpa II, and Not I.

[0067] The presence or absence of cleavage of a nucleic acid molecule comprising a target gene sequence by a methylation sensitive restriction endonuclease can be identified using any method useful for detecting the length or continuity of a polynucleotide sequence. For example, cleavage of the target gene sequence can be detected by Southern blot analysis, which allows mapping of the cleavage site, or using any other electrophoretic method or chromatographic method that separates nucleic acid molecules on the basis of relative size, charge, or a combination thereof. Cleavage of a target gene also can be detected using an oligonucleotide ligation assay, wherein, following contact with the

restriction endonuclease, a first oligonucleotide that selectively hybridizes upstream of and adjacent to a restriction endonuclease cleavage site and a second oligonucleotide that selectively hybridizes downstream of and adjacent to the cleavage site are contacted with the target gene sequence, and further contacted with a ligase such that, in the absence of cleavage the oligonucleotides are adjacent to each other and can be ligated together, whereas, in the absence of cleavage, ligation does not occur. By determining the size or other relevant parameter of the oligonucleotides following the ligation reaction, ligated oligonucleotides can be distinguished from unligated oligonucleotides, thereby providing an indication of restriction endonuclease activity.

[0068] Methylation silencing of a gene also can be detected by contacting a 5' regulatory region of the nucleic acid molecule comprising the gene of the test cell with a chemical reagent that selectively modifies either an unmethylated cytosine residue or a methylated cytosine residue, and detecting a product generated due to said contacting, wherein the product is indicative of methylation of a cytosine residue in a CpG dinucleotide of the gene, thereby detecting methylation silencing of the gene of the test cell. For example, the product can be detected using an electrophoresis method, a chromatography method, a mass spectrometry method, or a combination of such methods.

[0069] In one aspect of this embodiment, the gene is contacted with hydrazine, which modifies cytosine residues, but not methylated cytosine residues, then the hydrazine treated gene sequence is contacted with a reagent such as piperidine, which cleaves the nucleic acid molecule at hydrazine modified cytosine residues, thereby generating a product comprising fragments. By separating the fragments according to molecular weight, using, for example, an electrophoretic, chromatographic, or mass spectrographic method, and comparing the separation pattern with that of a similarly treated corresponding unmethylated gene sequence, gaps are apparent at positions in the test gene contained methylated cytosine residues. As such, the presence of gaps is indicative of methylation of a cytosine residue in the CpG dinucleotide in the target gene of the test cell.

[0070] In another aspect, a nucleic acid molecule comprising the target gene is contacted with a chemical reagent comprising bisulfite ions, for example, sodium bisulfite, which

converts unmethylated cytosine residues to bisulfite modified cytosine residues, then the bisulfite ion treated gene sequence is exposed to alkaline conditions, which convert bisulfite modified cytosine residues to uracil residues. Sodium bisulfite reacts readily with the 5,6-double bond of cytosine (but poorly with methylated cytosine) to form a sulfonated cytosine reaction intermediate that is susceptible to deamination, giving rise to a sulfonated uracil. As such, the sulfonate group can be removed by exposure to alkaline conditions, resulting in the formation of uracil. The DNA then can be amplified, for example, by PCR, and sequenced to determine the methylation status of all CpG sites. Uracil is recognized as a thymine by Taq polymerase and, upon PCR, the resultant product contains cytosine only at the position where 5-methylcytosine was present in the starting template DNA. By comparing the amount or distribution of uracil residues in the bisulfite ion treated gene sequence of the test cell with a similarly treated corresponding unmethylated gene sequence, detection of a decrease in the amount or distribution of uracil residues in the gene from the test cell is indicative of methylation of cytosine residues in CpG dinucleotides in the target gene of the test cell. The amount or distribution of uracil residues also can be detected by contacting the bisulfite ion treated target gene sequence, following exposure to alkaline conditions, with an oligonucleotide that selectively hybridizes to a nucleotide sequence of the target gene that either contains uracil residues or that lacks uracil residues, but not both, and detecting selective hybridization (or the absence thereof) of the oligonucleotide.

[0071] As used herein, the term "selective hybridization" or "selectively hybridize" or "specific hybridization" refers to an interaction of two nucleic acid molecules that occurs and is stable under moderately stringent or highly stringent conditions. As such, selective hybridization preferentially occurs, for example, between an oligonucleotide and a target nucleic acid molecule, and not substantially between the oligonucleotide and a nucleic acid molecule other than the target nucleic acid molecule, including not with nucleic acid molecules encoding related but different members of a gene family. Generally, an oligonucleotide useful as a probe or primer that selectively hybridizes to a target nucleic acid molecule is at least about 12 to 15 nucleotides in length, generally at least about 18 to 20 nucleotides in length, usually at least about 21 to 25 nucleotides in length, and particularly about 26 to 35 nucleotides in length or more. Examples of oligonucleotides useful in practicing the methods of the invention include those set forth as SEQ ID NOS:1

to 127, which are useful for examining gene listed in Table 2. Additional oligonucleotides useful for practicing the methods of the invention can be designed based on the present disclosure, and the nucleotide sequences available in the GenBank Accession numbers listed in Table 2.

[0072] Conditions that allow for selective hybridization can be determined empirically, or can be estimated based, for example, on the relative GC:AT (or GC:AU) content of the hybridizing oligonucleotide and the target nucleic acid molecule, the length of the hybridizing oligonucleotide, and the number, if any, of mismatches between the oligonucleotide and target sequence to which it is to hybridize (see, for example, Sambrook et al., "Molecular Cloning: A laboratory manual (Cold Spring Harbor Laboratory Press 1989)). As such, the conditions used to achieve a particular level of stringency will vary, depending on the nature of the hybridizing nucleic acid molecules. An additional consideration is whether one of the nucleic acids is immobilized, for example, on a filter. An example of progressively higher stringency conditions is as follows: 2X SSC/0.1% SDS at about room temperature (hybridization conditions); 0.2X SSC/0.1% SDS at about room temperature (low stringency conditions); 0.2X SSC/0.1% SDS at about 42°C (moderate stringency conditions); and 0.1X SSC at about 62°C (high stringency conditions). Hybridization and/or washing can be carried out using only one of these conditions, for example, high stringency conditions, or each of the conditions can be used, for example, for 10 to 15 minutes each, in the order listed above, repeating any or all of the steps listed.

[0073] Selective hybridization of an oligonucleotide with a target gene (e.g., a gene as listed in Table 2) can be detected, for example, by performing the method using an oligonucleotide that includes a detectable label. The detectable label can be any molecule that conveniently can be linked to the oligonucleotide and detected using readily available equipment. For example, the detectable label can be a fluorescent compound such as Cy3, Cy5, Fam, fluorescein, rhodamine, or a green fluorescent protein or enhanced or modified form thereof; a radionuclide such as sulfur-35, technicium-99, phosphorus-32, tritium or iodine-125; a paramagnetic spin label such as carbon-13, Gd-157, Mn-55, Dy-162, Cr-52, or Fe-56; a luminescent compound such as an aequorin; a chemiluminescent compound; a metal chelate; an enzyme such as luciferase or  $\beta$ -galactosidase, or a substrate for an

enzyme; or a receptor or a ligand for a receptor, for example, biotin. The means for detecting the detectable label will be selected based on the characteristics of the label, as will the means for linking the label to an oligonucleotide (see, for example, Hermanson, "Bioconjugate Techniques" (Academic Press 1996), which is incorporated herein by reference).

[0074] Selective hybridization also can be detected, for example, by utilizing the oligonucleotide as a substrate for a primer extension reaction, further contacting the sample with deoxyribonucleotides (dNTPs), including, if desired, a detectable dNTP (e.g., a fluorescently labeled dNTP, a digoxigenin labeled dNTP, or a biotin labeled dNTP), and a DNA dependent DNA polymerase under conditions sufficient for the primer extension reaction to proceed, and detecting a product of the primer extension reaction. Conditions for performing a primer extension reaction are well known in the art (see, for example, Sambrook et al., *supra*, 1989).

[0075] The amount or distribution of uracil residues in a bisulfite ion treated nucleic acid molecule comprising a target gene sequence following exposure to alkaline conditions also can be detected using an amplification reaction such as PCR. An amplification reaction is performed under conditions that allow selective hybridization of the forward and reverse primers of an amplification primer pair to the target nucleic acid molecule. Generally, the reaction is performed in a buffered aqueous solution, at about pH 7-9, usually about pH 8. In addition, the reaction generally is performed in a molar excess of primers to target nucleic acid molecule, for example, at a ratio of about 100:1 primer:genomic DNA. Where the amount of the target nucleic acid molecule in a sample is not known, for example, in a diagnostic procedure using a biological sample, a range of primer amounts can be used in samples run in parallel, although generally even the addition of a small amount of primers will result in a sufficient molar excess such that the amplification reaction can proceed.

[0076] The deoxyribonucleoside triphosphates, dATP, dCTP, dGTP, and dTTP, can be added to the synthesis mixture either separately or as a mixture, which can further include the primers, in adequate amounts and the resulting solution is heated to about 90°-100°C from about 1 to 10 minutes, preferably from 1 to 4 minutes. After this heating period, the

to 127, which are useful for examining gene listed in Table 2. Additional oligonucleotides useful for practicing the methods of the invention can be designed based on the present disclosure, and the nucleotide sequences available in the GenBank Accession numbers listed in Table 2.

[0072] Conditions that allow for selective hybridization can be determined empirically, or can be estimated based, for example, on the relative GC:AT (or GC:AU) content of the hybridizing oligonucleotide and the target nucleic acid molecule, the length of the hybridizing oligonucleotide, and the number, if any, of mismatches between the oligonucleotide and target sequence to which it is to hybridize (see, for example, Sambrook et al., "Molecular Cloning: A laboratory manual (Cold Spring Harbor Laboratory Press 1989)). As such, the conditions used to achieve a particular level of stringency will vary, depending on the nature of the hybridizing nucleic acid molecules. An additional consideration is whether one of the nucleic acids is immobilized, for example, on a filter. An example of progressively higher stringency conditions is as follows: 2X SSC/0.1% SDS at about room temperature (hybridization conditions); 0.2X SSC/0.1% SDS at about room temperature (low stringency conditions); 0.2X SSC/0.1% SDS at about 42°C (moderate stringency conditions); and 0.1X SSC at about 62°C (high stringency conditions). Hybridization and/or washing can be carried out using only one of these conditions, for example, high stringency conditions, or each of the conditions can be used, for example, for 10 to 15 minutes each, in the order listed above, repeating any or all of the steps listed.

[0073] Selective hybridization of an oligonucleotide with a target gene (e.g., a gene as listed in Table 2) can be detected, for example, by performing the method using an oligonucleotide that includes a detectable label. The detectable label can be any molecule that conveniently can be linked to the oligonucleotide and detected using readily available equipment. For example, the detectable label can be a fluorescent compound such as Cy3, Cy5, Fam, fluorescein, rhodamine, or a green fluorescent protein or enhanced or modified form thereof, a radionuclide such as sulfur-35, technicium-99, phosphorus-32, tritium or iodine-125; a paramagnetic spin label such as carbon-13, Gd-157, Mn-55, Dy-162, Cr-52, or Fe-56; a luminescent compound such as an aequorin; a chemiluminescent compound; a metal chelate; an enzyme such as luciferase or  $\beta$ -galactosidase, or a substrate for an

enzyme; or a receptor or a ligand for a receptor, for example, biotin. The means for detecting the detectable label will be selected based on the characteristics of the label, as will the means for linking the label to an oligonucleotide (see, for example, Hermanson, "Bioconjugate Techniques" (Academic Press 1996), which is incorporated herein by reference).

[0074] Selective hybridization also can be detected, for example, by utilizing the oligonucleotide as a substrate for a primer extension reaction, further contacting the sample with deoxyribonucleotides (dNTPs), including, if desired, a detectable dNTP (e.g., a fluorescently labeled dNTP, a digoxigenin labeled dNTP, or a biotin labeled dNTP), and a DNA dependent DNA polymerase under conditions sufficient for the primer extension reaction to proceed, and detecting a product of the primer extension reaction. Conditions for performing a primer extension reaction are well known in the art (see, for example, Sambrook et al., *supra*, 1989).

[0075] The amount or distribution of uracil residues in a bisulfite ion treated nucleic acid molecule comprising a target gene sequence following exposure to alkaline conditions also can be detected using an amplification reaction such as PCR. An amplification reaction is performed under conditions that allow selective hybridization of the forward and reverse primers of an amplification primer pair to the target nucleic acid molecule. Generally, the reaction is performed in a buffered aqueous solution, at about pH 7-9, usually about pH 8. In addition, the reaction generally is performed in a molar excess of primers to target nucleic acid molecule, for example, at a ratio of about 100:1 primer:genomic DNA. Where the amount of the target nucleic acid molecule in a sample is not known, for example, in a diagnostic procedure using a biological sample, a range of primer amounts can be used in samples run in parallel, although generally even the addition of a small amount of primers will result in a sufficient molar excess such that the amplification reaction can proceed.

[0076] The deoxyribonucleoside triphosphates, dATP, dCTP, dGTP, and dTTP, can be added to the synthesis mixture either separately or as a mixture, which can further include the primers, in adequate amounts and the resulting solution is heated to about 90°-100°C from about 1 to 10 minutes, preferably from 1 to 4 minutes. After this heating period, the

solution is allowed to cool to room temperature, which is preferable for the primer hybridization. To the cooled mixture is added an appropriate agent for effecting the primer extension reaction, generally a polymerase, and the reaction is allowed to occur under conditions as disclosed herein (see Example 1) or otherwise known in the art. Where the polymerase is heat stable, it can be added together with the other reagents. The polymerase can be any enzyme useful for directing the synthesis of primer extension products, including, for example, *E. coli* DNA polymerase I, Klenow fragment of *E. coli* DNA polymerase I, T4 DNA polymerase, other available DNA polymerases, polymerase muteins, reverse transcriptase, and other enzymes, including heat-stable enzymes, as are well known in the art and commercially available. The amplification products can be identified as methylated or non-methylated by a sequencing method, oligomer restriction (Saiki et al., *BioTechnology* 3:1008-1012, 1985), allele-specific oligonucleotide probe analysis (Conner et al., *Proc. Natl. Acad. Sci. USA* 80:278, 1983), oligonucleotide ligation assays (Landegren et al., *Science* 241:1077, 1988), and the like (see, also, Landegren et al., *Science* 242:229-237, 1988).

[0077] In one embodiment, the amplification is performed by contacting the target gene sequence (e.g., a gene as listed in Tables 2, 5 or 6) with an amplification primer pair comprising a forward primer and a reverse primer under conditions suitable for amplification, wherein at least one primer of the primer pair comprises an oligonucleotide that selectively hybridizes to a target gene sequence containing uracil residues, whereby generation of an amplification product is indicative of methylation of cytosine residues in CpG dinucleotides in the target gene of the test cell. In another embodiment, the amplification reaction is performed by contacting the target gene sequence with an amplification primer pair comprising a forward primer and a reverse primer under conditions suitable for amplification, wherein both primers of the primer pair selectively hybridize to a target gene sequence containing cytosine residues, but not to a target gene sequence containing uracil residues, whereby generation of an amplification product is indicative of a lack of methylation of cytosine residues in CpG dinucleotides in the target gene of the test cell.

[0078] In still another embodiment, a methylation-specific amplification reaction such as methylation-specific PCR (MSP) is used alone, or in combination with bisulfite treatment, to detect the methylation status of a nucleic acid molecule (see U.S. Pat. Nos. 6,265,171; 6,200,756; and 6,017,704, each of which is incorporated herein by reference; see, also, Example 1). MSP is a particularly sensitive method that allows detection of low numbers of methylated alleles and the use of small amounts of a nucleic acid sample, including paraffin-embedded materials, and also can be conveniently adapted to a multiplex analysis, including, for example, simultaneous detection of unmethylated and methylated products in a single sample, thus providing an internal control.

[0079] The amplification primer pairs used in an MSP reaction are designed to specifically distinguish between bisulfite untreated or unmodified DNA, and methylated and unmethylated DNA. MSP primer pairs for unmethylated DNA (unmethylation specific primer pairs) generally have a thymidine residue in the 3'-CpG pair to distinguish it from the cytosine residue retained in methylated DNA, and the complement is designed for the antisense primer. MSP primer pairs usually contain relatively few cytosine or guanine residues in the sequence because cytosine is absent in the sense (forward) primer and the guanine is absent in the antisense (reverse) primer; cytosine becomes modified to uracil, which is amplified as thymidine in the amplification product. MSP unmethylation specific primer pairs and MSP methylation specific primer pairs can be designed based on the nucleotide sequences set forth in the GenBank Accession numbers for the various genes listed in Table 2, including methylation-specific and unmethylation-specific primer pairs useful for amplification of a methylated or an unmethylated gene as listed in Table 2, for example, the ApoD methylation specific primer pair set forth as SEQ ID NOS:1 and 2, and the ApoD unmethylation specific primer pair set forth as SEQ ID NOS:3 and 4 (see, also, Table 4; SEQ ID NOS:65 to 127, disclosing methylation specific primer pairs for indicated genes, including primers used for bisulfite sequencing).

[0080] Accordingly, in one aspect, MSP is used for detecting the amount or distribution of uracil residues in a bisulfite ion treated target genes following alkaline treatment. Such a method can be performed by contacting the gene sequence with a first amplification primer pair and a second amplification primer pair under conditions suitable for amplification,

wherein the first amplification primer pair comprises a forward primer and a reverse primer, and at least one primer of the first primer pair comprises an oligonucleotide that selectively hybridizes to a nucleotide sequence of the target gene that contains uracil residues, and wherein the second amplification primer pair comprises a forward primer and a reverse primer, and both primers of the second primer pair selectively hybridize to a target gene containing cytosine residues, but not to a target gene sequence containing uracil residues, and wherein an amplification product, if any, generated by the first primer pair has a first length, and an amplification product, if any, generated by the second primer pair has a second length, which is different from the first length, whereby the length of the amplification products is indicative of the amount or distribution of uracil residues and, therefore, of methylation of cytosine residues in CpG dinucleotides in the target gene of the test cell.

[0081] The amount or distribution of uracil residues also can be detected by contacting the 5' regulatory region of the gene with a first amplification primer pair and a second amplification primer pair under conditions suitable for amplification, wherein the first amplification primer pair comprises a forward primer and a reverse primer, wherein at least one primer of the first primer pair comprises an oligonucleotide that selectively hybridizes to a nucleotide sequence of the 5' regulatory region of the gene containing uracil residues, and wherein the second amplification primer pair comprises a forward primer and a reverse primer, wherein both primers of the second primer pair selectively hybridize to a nucleotide sequence of the 5' regulatory region of the gene containing cytosine residues, but not to a corresponding nucleotide sequence of the 5' regulatory region of the gene containing uracil residues, and wherein an amplification product, if any, generated by the first primer pair has a first length, and wherein an amplification product, if any, generated by the second primer pair has a second length, which is different from the first length, whereby the length of the amplification products is indicative of uracil residues and, therefore, methylation of cytosine residues in CpG dinucleotides in the 5' regulatory region of the gene, thereby detecting methylation silencing of the gene of the test cell.

[0082] Methylation silencing of a gene in a cell exhibiting or suspected of exhibiting unregulated growth (e.g., a gene associated with a cancer) also can be identified by

contacting a test cell with a demethylating agent, and detecting increased expression of an RNA encoded by the gene as compared to a level of expression of the RNA in a test cell not contacted with a demethylating agent. Such a method can further include detecting methylation, if any, of cytosine residues in a CpG dinucleotide in a CpG island of the 5' regulatory region of the gene in a corresponding cell exhibiting regulated growth, or an extract of the corresponding cell. The demethylating agent can be a methyltransferase inhibitor such as 5Aza-dC. Increased expression of an RNA can be detected using any method for detecting RNA, including, for example, northern blot analysis, a reverse transcription-polymerase chain reaction assay, or selective hybridization to an array of nucleotide sequences as disclosed herein. Accordingly, the methods of the invention can be performed in a high throughput format, wherein the test cell, or extract of the test cell, comprises one of a plurality of test cells, or extracts of the test cells, or a combination thereof; and each of the test cells, or extracts of the test cells, of the plurality is the same or different, or a combination thereof.

[0083] In adapting the methods of the invention to a high throughput format, the test cells, or extracts of the test cell, can be arranged in an array, which can be an addressable array, on a solid support such as a microchip, a glass slide, or a bead, and the cells (or extracts) can be contacted serially or in parallel with an oligonucleotide probe or primer (or primer pair) as disclosed herein. Samples arranged in an array or other reproducible pattern can be assigned an address (i.e., a position on the array), thus facilitating identification of the source of the sample. An additional advantage of arranging the samples in an array, particularly an addressable array, is that an automated system can be used for adding or removing reagents from one or more of the samples at various times, or for adding different reagents to particular samples. In addition to the convenience of examining multiple samples at the same time, such high throughput assays provide a means for examining duplicate, triplicate, or more aliquots of a single sample, thus increasing the validity of the results obtained, and for examining control samples under the same conditions as the test samples, thus providing an internal standard for comparing results from different assays. Conveniently, cells or extracts at a position in the array can be contacted with two or more oligonucleotide probes or primers (or primer pairs), wherein the oligonucleotides are differentially labeled or comprise a reaction that generates distinguishable products, thus

providing a means for performing a multiplex assay. Such assays can allow the examination of one or more, particularly 2, 3, 4, 5, 10, 15, 20, or more genes to identify epigenetically silenced genes in a test cell.

[0084] The present invention also provides oligonucleotides, which can be useful as probes or primers for identifying an epigenetic silenced gene (or the absence thereof). As used herein, the term "oligonucleotide", "polynucleotide", or "nucleic acid molecule" is used broadly to mean a sequence of two or more deoxyribonucleotides or ribonucleotides that are linked together by a phosphodiester bond. The term "gene" also is used herein to refer to a polynucleotide sequence contained in a genome. It should be recognized, however, that a nucleic acid molecule comprising a portion of a gene can be isolated from a cell or can be examined as genomic DNA, for example, by a hybridization reaction or a PCR reaction. Thus, while in a genome, it may not always be clear as to a specific nucleotide position where a gene begins or ends, for purposes of the present invention, a gene is considered to be a discrete nucleic acid molecule that includes at least the nucleotide sequence set forth in the GenBank Accession Numbers shown in Tables 2, 5 and 6, for various genes identified and or examined herein.

[0085] For convenience of discussion, the term "oligonucleotide" is used herein to refer to a polynucleotide that is used as a probe or primer, whereas the term "polynucleotide" or "nucleic acid molecule" is used more broadly to encompass any sequence of two or more nucleotides, including an oligonucleotide. In addition, the term "nucleotide sequence" is used to refer to the molecules that are present on an array. As such, it should be recognized that the various terms used herein to conveniently distinguish different nucleic acid molecules. As such, the terms include RNA and DNA, which can be a gene or a portion thereof, a cDNA, a synthetic polydeoxyribonucleic acid sequence, or the like. Generally, an oligonucleotide or polynucleotide can be single stranded or double stranded, as well as a DNA/RNA hybrid, although it will be recognized that the strands of a double stranded oligonucleotide that is to be used as a probe or primer will be separated, for example, by heating a solution containing the oligonucleotide above the melting temperature of the particular oligonucleotide.

[0086] The terms "oligonucleotide", "polynucleotide", and the like as used herein include naturally occurring nucleic acid molecules, which can be isolated from a cell, as well as fragments thereof as produced, for example, by a restriction endonuclease digestion, and synthetic molecules, which can be prepared, for example, by methods of chemical synthesis or by enzymatic methods such as by PCR. In various embodiments, an oligonucleotide or polynucleotide of the invention can contain nucleoside or nucleotide analogs, or a backbone bond other than a phosphodiester bond, for example, a thiodiester bond, a phosphorothioate bond, a peptide-like bond or any other bond known to those in the art as useful for linking nucleotides to produce synthetic polynucleotides (see, for example, Tam et al., *Nucl. Acids Res.* 22:977-986, 1994); Ecker and Crooke, *BioTechnology* 13:351360, 1995, each of which is incorporated herein by reference). The incorporation of non-naturally occurring nucleotide analogs or bonds linking the nucleotides or analogs can be particularly useful where the polynucleotide is to be exposed to an environment that can contain a nucleolytic activity, including, for example, a tissue culture medium, a cell or in a living subject, since the modified polynucleotides can be designed to be less (or, if desired, more) susceptible to degradation.

[0087] In general, the nucleotides comprising a polynucleotide are naturally occurring deoxyribonucleotides, such as adenine, cytosine, guanine or thymine linked to 2'-deoxyribose, or ribonucleotides such as adenine, cytosine, guanine or uracil linked to ribose. However, a polynucleotide (or oligonucleotide) also can contain nucleotide analogs, including non-naturally occurring synthetic nucleotides or modified naturally occurring nucleotides. Such nucleotide analogs are well known in the art and commercially available, as are polynucleotides containing such nucleotide analogs (Lin et al., *Nucl. Acids Res.* 22:5220-5234, 1994; Jellinek et al., *Biochemistry* 34:11363-11372, 1995; Pagratis et al., *Nature Biotechnol.* 15:68-73, 1997, each of which is incorporated herein by reference).

[0088] A polynucleotide comprising naturally occurring nucleotides and phosphodiester bonds can be chemically synthesized or can be produced using recombinant DNA methods, using an appropriate polynucleotide as a template. In comparison, a polynucleotide comprising nucleotide analogs or covalent bonds other than phosphodiester bonds generally will be chemically synthesized, although an enzyme such as T7 polymerase can incorporate

certain types of nucleotide analogs into a polynucleotide and, therefore, can be used to produce such a polynucleotide recombinantly from an appropriate template (Jellinek et al., *supra*, 1995). As such, the polynucleotide can be prepared using a method such as conventional phosphotriester and phosphodiester methods, including, for example, an automated method such as that using diethylphosphoramidites (see Beaucage et al., *Tetrahedron Lett.*, 22:1859-1862, 1981), or a method whereby the oligonucleotides are synthesized on a modified solid support (see U.S. Pat. No. 4,458,066).

[0089] An oligonucleotide of the invention, which can selectively hybridize to a target nucleic acid molecule and can be used as a reagent for detecting expression and/or methylation (or lack of methylation; "unmethylation") of a gene, is designed to selectively hybridize to a nucleotide sequence within about 2000 nucleotides upstream (5') or downstream (3') of the target gene, and generally within about 1000 nucleotides of the region comprising the CpG island that is to be examined for cytosine methylation, usually within about 500 nucleotides of the site to be examined. In addition, an oligonucleotide of the invention, or useful in a method of the invention, is at least about 12 nucleotides in length, generally at least about 14 or 15 nucleotides in length, usually at least about 18 to 20 nucleotides, and can be about 25, 30, 35 or more nucleotides in length, such that it can selectively hybridize to a target nucleic acid molecule. It will be recognized that the length of the oligonucleotide will depend, in part, on the target gene. For example, when the target gene is one of a family of closely related genes having regions of substantial sequence similarity, a longer oligonucleotide can be used to assure selective hybridization to the target gene and minimal, if any, cross-hybridization to the related gene sequence(s).

[0090] Oligonucleotides of the invention are designed to be substantially complementary to at least one strand of a double stranded nucleic acid molecule corresponding to a genomic locus (to each of both strands where an intervening sequence is to be amplified) and, where they are to be used for differentiating methylated from unmethylated cytosine residues, will include the appropriate guanine or cytosine residues, as discussed above. Oligonucleotides of the invention are exemplified by amplification primer pairs useful for RT-PCR of a nucleotide sequence of a target gene (see Table 3; SEQ ID NOS:7 to 64); and for methylation specific or unmethylation specific amplification of a nucleotide sequence of a

target gene; or for bisulfite PCR (see Table 4; SEQ ID NOS:65 to 127, methylation specific primers; SEQ ID NO.

[0091] Accordingly, the present invention provides an oligonucleotide selected from any one of SEQ ID NOS:1 to 127, and further provides a plurality of such oligonucleotides, which includes at least two (e.g., 2, 3, 4, or more) of the oligonucleotides set forth as SEQ ID NOS:1 to 127, including, for example, a combination comprising at least two oligonucleotides useful as an amplification primer pair, which can amplify a portion of an ApoD gene as listed in Table 2, in some cases depending, for example, on whether the target sequence is methylated or unmethylated. The present invention also provides an amplification primer pair, which comprises a forward primer and a reverse primer, particularly a primer pair that includes one, and particularly two, oligonucleotides, which can be a forward primer, a reverse primer or both of a primer pair, particularly a primer pair useful for amplifying a portion of a gene as listed in Table 2. In one aspect, an amplification primer pair of the invention can be used to specifically amplify a methylated 5' regulatory region of the nucleic acid molecule. In another aspect, an amplification primer pair of the invention can be used to specifically amplify an unmethylated 5' regulatory region of the nucleic acid molecule.

[0092] The present invention also relates to a kit, which contains at least one isolated oligonucleotide of the invention, including, for example, a plurality of such isolated oligonucleotides. In one embodiment, a plurality of isolated oligonucleotides of a kit of the invention includes at least one amplification primer pair (i.e., a forward primer and a reverse primer), and can include a plurality of amplification primer pairs, including, for example, amplification primer pairs as disclosed herein. As such, a kit of the invention can contain, for example, one or a plurality of methylation specific amplification primer pairs, unmethylation specific amplification primer pairs, or a combination methylation specific amplification primer pairs and unmethylation specific amplification primer pair, including methylation specific primer pairs and unmethylation specific primer pairs useful for amplifying a methylated form or an unmethylated form of a particular gene that is known to be or suspected of being methylation silenced in one or more types of cancer cells.

[0093] A kit of the invention can further include additional reagents, which can be useful, for example, for a purpose for which the oligonucleotides of the kit are useful. For example, where a kit contains one or a plurality of methylation specific and/or unmethylation specific amplification primers, the kit can further contain, for example, control polynucleotides, which can be methylated or unmethylated; one or more reagents that modify methylated cytosine residues, and/or one or more reagents for performing an amplification reaction. Where the kit contains one or plurality of oligonucleotides that selectively hybridize to a methylated or to an unmethylated gene sequence, the kit can further contain, for example, a methylation sensitive restriction endonuclease. A kit of the invention also can contain at least a second primer pair, which can, but need not, be one of the above listed primer pairs, and can be useful, for example, for a nested amplification reaction. Such additional primer pairs can be designed based on the expected sequence of the amplified portion of the target gene using the sequence information available in the relevant GenBank Accession No. for the target gene (see Table 2).

[0094] In one embodiment, a kit of the invention contains a methylation specific primer pair and an unmethylation specific primer pair, which are specific for the same target gene, thus allowing a user of the kit to determine whether a particular target gene is methylated or unmethylated. In another embodiment, the kit contains a plurality of such methylation specific and unmethylation specific primer pairs, thus allowing a user to determine the methylation of one or more target genes. For example, such a kit can contain a methylation specific primer pair and an unmethylation specific primer pair for one or more selected target genes, e.g., an ApoD gene, an NU gene, a CRIP1 gene, or other gene as set forth in Table 2, thus providing amplification primer pairs useful for determining whether the 5' regulatory region of one or more selected genes is methylated or unmethylated. Such a kit can further contain a primer pair that includes oligonucleotides that selectively hybridize to an expected amplification product generated using the methylation specific or unmethylation specific primer pair, thus providing reagents useful for performing a nested amplification procedure.

[0095] A kit of the invention also can contain a detectable label that can be linked to or incorporated into an oligonucleotide of the kit, or a plurality of different detectable labels

such that, depending the needs of the user, can be selected for a particular use, and, if desired, reagents for linking or incorporating the detectable label into the oligonucleotide. Alternatively, or in addition, the kit can contain one or more reagents useful for performing a hybridization reaction such that selective hybridization conditions readily are attained; and/or can contain one or more standard nucleic acid molecules, for example, a standard target ApoD gene nucleotide sequence that contains methylated cytosine residues corresponding the region to which the oligonucleotide is designed to selectively hybridize, or a standard target ApoD gene nucleotide sequence that contains unmethylated cytosine residues corresponding to the target sequence, or a combination thereof. Such standards provide several advantages, including, for example, allowing a confirmation that a reaction using a test cell, or extract thereof, functioned properly, or allowing for comparisons among samples examined at different times or collected from different sources.

[0096] Where a kit contains one or more oligonucleotides useful for performing a primer extension (or amplification) reaction, the kit can further include reagents for performing the selective hybridization reaction such that the oligonucleotide provides a substrate for the extension reaction; and/or one or more reagents for performing the primer extension (or amplification) reaction, for example, dNTPs, one or more of which can be detectably labeled or otherwise modified for conveniently linking a detectable label; one or a selection of polymerases; and/or one or more standard target nucleic acid molecules. Where a kit of the invention contains two or more oligonucleotides (or primer pairs) such as those exemplified herein or otherwise useful for practicing the methods of the invention, the kit provides a convenient source of reagents from which the skilled artisan can select one or more oligonucleotides (or primer pairs), as desired.

[0097] The present invention also relates to a method of reducing or inhibiting unregulated growth of a cell exhibiting epigenetic silenced transcription of at least one gene associated with a cancer. Such a method can be practiced, for example, by restoring expression of a polypeptide encoded by the epigenetic silenced gene in the cell, thereby reducing or inhibiting unregulated growth of the cell. In one embodiment, expression of the polypeptide encoded by the epigenetic silenced gene can be restored by contacting the cell with a demethylating agent, a histone deacetylase inhibitor, or a combination thereof. In

one aspect of this embodiment, at least one epigenetic silenced gene comprises a methylation silenced gene, and the cells are contacted with a demethylating agent such as 5Aza-dC, for example, by administering the demethylating agent locally or systemically to the subject such that it contacts the cells *in vivo*.

[0098] A method of restoring expression of a polypeptide encoded by the epigenetic silenced gene in a cell also can be performed by introducing a polynucleotide encoding the polypeptide into the cell, whereby the polypeptide is expressed from the polynucleotide. The polynucleotide can, but need not, be contained in a vector, for example, a viral vector, and can be formulated, for example, in a matrix such as a liposome, microbubbles, or the like. The polynucleotide can be introduced into a cell by administering the polynucleotide to the subject such that it contacts the cell, wherein it can be taken up by the cell and the encoded polypeptide expressed.

[0099] A polynucleotide useful in such a method can be any polynucleotide corresponding to an epigenetically silenced gene. For example, where the cell is an ESCC cell, the epigenetic silenced gene can be a gene as set forth in Table 2, and the polynucleotide can be a nucleic acid molecule encoding a polypeptide encoded by the gene, such polynucleotides being available at the GenBank Accession No. as indicated in Table 2. For example, the epigenetic silenced gene can be an ApoD, NU, swisprosin-2, Hep27, KIF5C, keratin 14, transglutaminase 2, MUC1, IL-1 R2, crystallin alpha2, CLF-1, CRIP-1, Rad, HEM45, KLF6, follistatin related protein FLRG, XAP-5, Tbc1d1, cyclin G1 interacting protein, CRBP, metallothionein 1G, claudin-3, uncoupling protein-2, or apolipoprotein C1 gene, or a combination thereof.

[0100] In one embodiment, the cell is an ESCC cell, and the epigenetic silenced gene comprises a methylation silenced gene, for example, a methylation silenced ApoD, NU, CLF-1, CRIP-1, claudin-3, uncoupling protein-2, metallothionein 1G, transglutaminase 2, or apolipoprotein C1 gene, or a combination thereof. In another embodiment, the cell is an ESCC cell, and the epigenetic silenced gene comprises a tumor suppressor gene, for example, an ApoD, NU, or CRIP-1 gene; or the tumor suppressor gene comprises a

neuromedin B, or receptor of G protein signaling 2 (RGS2) gene; or the ESCC cell contains a combination of such tumor suppressor genes.

[0101] The present invention further relates to a method for treating a cancer patient, wherein cancer cells in the patient exhibit epigenetic silenced expression of at least one gene. Such a method can be performed, for example, by restoring expression of the at least one epigenetic silenced gene in cancer cells in the subject, thereby treating the cancer patient. At least one epigenetic silenced gene can be a methylation silenced gene, and can, but need not, be a tumor suppressor gene or a gene that affects the activity or expression of a tumor suppressor gene.

[0102] In one embodiment, cancer cells of the cancer patient contain at least one methylation silenced gene, and the method comprises administering a demethylating agent to the subject in an amount sufficient to restore expression of the methylation silenced gene in cancer cells in the subject. In another embodiment, cancer cells of the cancer patient contain at least one epigenetic silenced gene, and the method comprises administering at least one polynucleotide encoding a polypeptide encoded by an epigenetic silenced gene to the subject under conditions sufficient for expression of the at least one polypeptide in cancer cells in the subject. The polynucleotide can be contained in a vector such as a viral vector; and/or can be formulated with a matrix such as liposomes or microbubbles.

[0103] A cancer treated according to a method of the invention can be any cancer comprising cancer cells containing at least one epigenetic silenced gene associated with the cancer, including, for example, a carcinoma or a sarcoma. In one embodiment, the cancer is an esophageal squamous cell carcinoma, and the epigenetic silenced gene includes one or more genes as set forth in Table 2. For example, the epigenetic silenced gene can be an ApoD, NU, swisprosin-2, Hep27, KIF5C, keratin 14, transglutaminase 2, MUC1, IL-1 R2, crystallin alpha2, CLF-1, CRIP-1, Rad, HEM45, KLF6, follistatin related protein FLRG, XAP-5, Tbc1d1, cyclin G1 interacting protein, CRBP, metallothionein 1G, claudin-3, uncoupling protein-2, or apolipoprotein C1 gene, or a combination thereof. In one aspect, the epigenetic silenced gene(s) include at least one methylation silenced gene, for example, an ApoD, NU, CLF-1, CRIP-1, claudin-3, uncoupling protein-2, metallothionein 1G,

transglutaminase 2, or apolipoprotein C1 gene, or a combination thereof. In another aspect, the epigenetic silenced gene(s) include at least one tumor suppressor gene, for example, an ApoD, NU, and/or CRIP-1 gene; or a neuromedin B and/or RGS2 gene; or a combination thereof.

[0104] The present invention also relates to a method for selecting a therapeutic strategy for treating a cancer patient. Such a method can be performed, for example, by identifying at least one epigenetically silenced gene associated with the cancer according to a genome screening method of the invention as disclosed herein; and selecting an agent useful for restoring expression of the at least one epigenetically silenced gene in cancer cells of the patient. The agent can be, for example, a polynucleotide encoding a polypeptide otherwise expressed from the epigenetically silenced gene(s), for example, a polynucleotide encoding a polypeptide encoded by a gene listed in Table 2 such as an ApoD, NU, swisprosin-2, Hep27, KIF5C, keratin 14, transglutaminase 2, MUC1, IL-1 R2, crystallin alpha2, CLF-1, CRIP-1, Rad, HEM45, KLF6, follistatin related protein FLRG, XAP-5, Tbc1d1, cyclin G1 interacting protein, CRBP, metallothionein 1G, claudin-3, uncoupling protein-2, or apolipoprotein C1 gene, or a combination thereof.

[0105] In one embodiment, the identified epigenetic silenced gene comprises at least one methylation silenced gene, and the agent selected is one useful for restoring expression of the at least one methylation silenced gene in the cancer cells. In one aspect of this method, the selected agent comprises a polynucleotide encoding a polypeptide otherwise encoded by the methylation silenced gene(s), for example, an ApoD, NU, CLF-1, CRIP-1, claudin-3, uncoupling protein-2, metallothionein 1G, transglutaminase 2, or apolipoprotein C1 gene. In another aspect of this method, the selected agent comprises a demethylating agent, for example, 5Aza-dC.

[0106] In another embodiment, the identified epigenetic silenced gene comprises at least one tumor suppressor gene, and the agent selected is one useful for restoring a polypeptide encoded by the epigenetic silenced tumor suppressor gene in the cancer cells. For example, the tumor suppressor gene can be an ApoD, NU, or CRIP-1 gene, or a neuromedin B or

RGS2 gene, or a combination of such genes, and the selected agent can be a polynucleotide encoding an ApoD, NU, CRIP-1, neuromedin B, and/or RGS2 gene product.

[0107] The present invention also relates to a method of treating a subject suffering from an ESCC, wherein cells associated with the ESCC contain at least one epigenetic silenced gene. Such a method can be performed, for example, by administering an amount of an agent that restores expression of the at least one epigenetic silenced gene to the subject sufficient to restore expression of the epigenetic silenced gene in cells associated with the ESCC, thereby treating the subject. In one embodiment, the agent comprises a polynucleotide encoding the at least one epigenetically silenced gene, particularly a polynucleotide comprising a coding sequence of a gene as listed in Table 2, for example, a polynucleotide comprising a coding sequence of an ApoD, NU, swisprosin-2, Hep27, KIF5C, keratin 14, transglutaminase 2, MUC1, IL-1 R2, crystallin alpha2, CLF-1, CRIP-1, Rad, HEM45, KLF6, follistatin related protein FLRG, XAP-5, Tbc1d1, cyclin G1 interacting protein, CRBP, metallothionein 1G, claudin-3, uncoupling protein-2, or apolipoprotein C1 gene, or a combination thereof.

[0108] In another embodiment, at least one epigenetic silenced gene is a methylation silenced gene, and the agent for treating the subject comprises a polynucleotide encoding a polypeptide encoded by the methylation silenced gene. For the polynucleotide can comprises a coding sequence of an ApoD, NU, CLF-1, CRIP-1, claudin-3, uncoupling protein-2, metallothionein 1G, transglutaminase 2, or apolipoprotein C1 gene, or a combination thereof. In still another embodiment, at least one epigenetically silenced gene comprises at least one tumor suppressor gene, and the method of treating a subject comprises restoring expression of the tumor suppressor gene in ESCC cells of the subject. For example, the tumor suppressor gene can be an ApoD, NU, or CRIP-1; or can be a neuromedin B, or RGS2 gene; or can be a combination including at least one of such genes; and the agent can be a polynucleotide encoding one or more of the epigenetically silenced tumor suppressor genes. An agent useful in a method of the invention can be administered to the subject locally or systemically, such that it can contact the ESCC cells in the subject.

[0109] As a result of methylation silenced transcription of one or more genes in a cell, the gene product(s) is not present in the cell and, therefore, there is a loss of function associated with the absence of the encoded gene product(s). For example, epigenetic silencing of the ApoD gene, which is associated with cell growth arrest, results in loss of this function and, therefore, unregulated cell growth. Accordingly, the methods of the invention are based on providing a cell that exhibits unregulated growth due to epigenetic silenced, particularly methylation silenced, gene expression with the polypeptide encoded by the methylation silenced gene, thereby restoring regulated growth to the cell. As disclosed herein, the polypeptide can be provided to the cell directly, can be expressed from an exogenous polynucleotide that is introduced into the cell and encodes the polypeptide, or by restoring expression of the endogenous methylation silenced gene in the cell. By restoring the polypeptide to a cell exhibiting unregulated growth, or characteristics generally associated with unregulated growth, including, for example, the ability to grow in soft agar, a lack of contact inhibited growth, or refractoriness to programmed cell death, are alleviated.

[0110] Expression of one or more methylation silenced genes such as one or more genes shown in Table 2 can be restored, for example, by contacting the cells with a demethylating agent such as 5Aza-dC, which, when incorporated into the genes during replication of the cell results in progeny cells containing unmethylated genes, which can be transcribed. If desired, prior to administration to a subject, a sample of the target cells from the subject (or cells corresponding to the target cells) can be contacted with the demethylating agent in culture to determine or confirm that the demethylating agent is provided in an amount sufficient to result in demethylation of the target genes, without being toxic to the cells. The cells contacted in culture generally are cells of the subject, which are being examined prior to administration of the demethylating agent to the subject, but also can be, for example, cells of an established cell line that are of the same type as those to be contacted in the subject, e.g., ESCC cells. A method of treating a subject according to the present invention can further include treating the subject with agents otherwise known in the art as useful for treating a subject having the particular cancer, or that can be newly useful when used in combination with the present methods.

[0111] Cells exhibiting methylation silenced gene expression generally are contacted with the demethylating agent *in vivo* by administering the agent to a subject. Where convenient, the demethylating agent can be administered using, for example, a catheterization procedure, at or near the site of the cells exhibiting unregulated growth in the subject, or into a blood vessel in which the blood is flowing to the site of the cells. Similarly, where an organ, or portion thereof, to be treated can be isolated by a shunt procedure, the agent can be administered via the shunt, thus substantially providing the agent to the site containing the cells. The agent also can be administered systemically or via other routes as disclosed herein or otherwise known in the art.

[0112] A polypeptide, which is reduced or absent due to an epigenetic silenced gene, also can be provided to a cell by introducing a polynucleotide encoding the polypeptide into the cell, whereby the polypeptide is expressed from the polynucleotide in the cell. As such, the present invention provides methods of gene therapy. For example, where the cell is characterized by methylation silenced transcription of the ApoD gene, a polynucleotide having a nucleotide sequence as set forth in GenBank Accession No. J0261 (see Table 2) can be introduced into the target cell.

[0113] The polynucleotide can include, in addition to polypeptide coding sequence, operatively linked transcriptional regulatory elements, translational regulatory elements, and the like, and can be in the form of a naked DNA molecule, which can be contained in a vector, or can be formulated in a matrix such as a liposome or microbubbles that facilitates entry of the polynucleotide into the particular cell. As used herein, the term "operatively linked" refers to two or more molecules that are positioned with respect to each other such that they act as a single unit and effect a function attributable to one or both molecules or a combination thereof. For example, a polynucleotide encoding an ApoD polypeptide can be operatively linked to a second (or more) coding sequence, such that a chimeric polypeptide can be expressed from the operatively linked coding sequences. The chimeric polypeptide can be a fusion protein, in which the two (or more) encoded polypeptides are translated into a single polypeptide, i.e., are covalently bound through a peptide bond; or can be translated as two discrete peptides that, upon translation, can operatively associate with each other to form a stable complex. Similarly, a polynucleotide sequence encoding a desired

polypeptide can be operatively linked to a regulatory element, in which case the regulatory element confers its regulatory effect on the polynucleotide similarly to the way in which the regulatory element would effect a polynucleotide sequence with which it normally is associated with in a cell.

[0114] A fusion protein generally demonstrates some or all of the characteristics of each of its polypeptide components, and, therefore, can be useful for restoring gene expression in the cell and can further provide additional advantages. For example, the fusion protein can include a polypeptide, which is otherwise reduced or absent due to epigenetic silencing of its encoding gene, operatively linked to a cell compartment localization domain such that expression of the fusion protein in a cell or loading of the cell with fusion protein allows translocation of the encoded polypeptide to the intracellular compartment such as the nucleus, in which it effects its activity. Cell compartmentalization domains, for example, are well known and include a plasma membrane localization domain, a nuclear localization signal, a mitochondrial membrane localization signal, an endoplasmic reticulum localization signal, and the like, as well as signal peptides, which can direct secretion of a polypeptide from a cell (see, for example, Hancock et al., *EMBO J.* 10:4033-4039, 1991; Buss et al., *Mol. Cell. Biol.* 8:3960-3963, 1988; U.S. Pat. No. 5,776,689 each of which is incorporated herein by reference). The fusion protein also can comprise a desired polypeptide operatively linked to a peptide that acts as a ligand for a receptor, a peptide useful as a tag for identifying a cell in which the polypeptide is expressed, or for isolating the fusion protein, or any other peptide or polypeptide of interest, providing the fusion protein has the protein activity of the desired polypeptide. Peptide tags such as a polyhistidine tag peptide, e.g., His-6, which can be detected using a divalent cation such as nickel ion, cobalt ion, or the like; a FLAG epitope, which can be detected using an anti-FLAG antibody (see, for example, Hopp et al., *BioTechnology* 6:1204 (1988); U.S. Pat. No. 5,011,912, each of which is incorporated herein by reference); a c-myc epitope, which can be detected using an antibody specific for the epitope; biotin, which can be detected using streptavidin or avidin; and glutathione S-transferase, which can be detected using glutathione, are well known in the art, and provide a means of detecting the presence of a polypeptide operatively linked thereto. Such tags provide the additional advantage that they can facilitate isolation of the operatively linked polypeptide, for example, where it is desired to obtain the polypeptide in a

substantially purified form, such a polypeptide also being useful for practicing methods of the invention.

[0115] A polynucleotide encoding a polypeptide otherwise encoded by an epigenetic silenced can be used alone, or can be contained in a vector, which can facilitate manipulation of the polynucleotide, including introduction of the polynucleotide into a target cell. The vector can be a cloning vector, which is useful for maintaining the polynucleotide, or can be an expression vector, which contains, in addition to the polynucleotide, regulatory elements useful for expressing the polynucleotide and encoded polypeptide in a particular cell. An expression vector can contain the expression elements necessary to achieve, for example, sustained transcription of the encoding polynucleotide, or the regulatory elements can be operatively linked to the polynucleotide prior to its being cloned into the vector.

[0116] An expression vector (or the polynucleotide encoding the desired polypeptide) generally contains or encodes a promoter sequence, which can provide constitutive or, if desired, inducible or tissue specific or developmental stage specific expression of the encoding polynucleotide, a poly-A recognition sequence, and a ribosome recognition site or internal ribosome entry site, or other regulatory elements such as an enhancer, which can be tissue specific. The vector also can contain elements required for replication in a prokaryotic or eukaryotic host system or both, as desired. Such vectors, which include plasmid vectors and viral vectors such as bacteriophage, baculovirus, retrovirus, lentivirus, adenovirus, vaccinia virus, semliki forest virus and adeno-associated virus vectors, are well known and can be purchased from a commercial source (Promega, Madison WI; Stratagene, La Jolla CA; GIBCO/BRL, Gaithersburg MD) or can be constructed by one skilled in the art (see, for example, *Meth. Enzymol.*, Vol. 185, Goeddel, ed. (Academic Press, Inc., 1990); Jolly, *Canc. Gene Ther.* 1:51-64, 1994; Flotte, *J. Bioenerg. Biomemb.* 25:37-42, 1993; Kirshenbaum et al., *J. Clin. Invest.* 92:381-387, 1993; each of which is incorporated herein by reference).

[0117] A tetracycline (tet) inducible promoter can be particularly useful for driving expression of a polynucleotide encoding a desired polypeptide. Upon administration of

tetracycline, or a tetracycline analog, to a subject containing a polynucleotide operatively linked to a tet inducible promoter, expression of the encoded polypeptide is induced. The polynucleotide also can be operatively linked to tissue specific regulatory element, for example, a liver cell specific regulatory element such as an  $\alpha$ -fetoprotein promoter (Kanai et al., *Cancer Res.* 57:461-465, 1997; He et al., *J. Exp. Clin. Cancer Res.* 19:183-187, 2000) or an albumin promoter (Power et al., *Biochem. Biophys. Res. Comm.* 203:1447-1456, 1994; Kuriyama et al., *Int. J. Cancer* 71:470-475, 1997); a muscle cell specific regulatory element such as a myoglobin promoter (Devlin et al., *J. Biol. Chem.* 264:13896-13901, 1989; Yan et al., *J. Biol. Chem.* 276:17361-17366, 2001); a prostate cell specific regulatory element such as the PSA promoter (Schuur et al., *J. Biol. Chem.* 271:7043-7051, 1996; Latham et al., *Cancer Res.* 60:334-341, 2000); a pancreatic cell specific regulatory element such as the elastase promoter (Ornitz et al., *Nature* 313:600-602, 1985; Swift et al., *Genes Devel.* 3:687-696, 1989); a leukocyte specific regulatory element such as the leukosialin (CD43) promoter (Shelley et al., *Biochem. J.* 270:569-576, 1990; Kudo and Fukuda, *J. Biol. Chem.* 270:13298-13302, 1995); or the like, such that expression of the polypeptide is restricted to particular cell in an individual, or to particular cells in a mixed population of cells in culture, for example, an organ culture. Regulatory elements, including tissue specific regulatory elements, many of which are commercially available, are well known in the art (see, for example, InvivoGen; San Diego CA).

[0118] Viral expression vectors can be particularly useful for introducing a polynucleotide into a cell, particularly a cell in a subject. Viral vectors provide the advantage that they can infect host cells with relatively high efficiency and can infect specific cell types. For example, a polynucleotide encoding a desired polypeptide can be cloned into a baculovirus vector, which then can be used to infect an insect host cell, thereby providing a means to produce large amounts of the encoded polypeptide. The viral vector also can be derived from a virus that infects cells of an organism of interest, for example, vertebrate host cells such as mammalian, avian or piscine host cells. Viral vectors can be particularly useful for introducing a polynucleotide useful in performing a method of the invention into a target cell. Viral vectors have been developed for use in particular host systems, particularly mammalian systems and include, for example, retroviral vectors, other lentivirus vectors such as those based on the human immunodeficiency virus (HIV),

adenovirus vectors, adeno-associated virus vectors, herpesvirus vectors, hepatitis virus vectors, vaccinia virus vectors, and the like (see Miller and Rosman, *BioTechniques* 7:980-990, 1992; Anderson et al., *Nature* 392:25-30 Suppl., 1998; Verma and Somia, *Nature* 389:239-242, 1997; Wilson, *New Engl. J. Med.* 334:1185-1187 (1996), each of which is incorporated herein by reference).

[0119] A polynucleotide, which can be contained in a vector, can be introduced into a cell by any of a variety of methods known in the art (Sambrook et al., *supra*, 1989; Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, Baltimore, MD (1987, and supplements through 1995), each of which is incorporated herein by reference). Such methods include, for example, transfection, lipofection, microinjection, electroporation and, with viral vectors, infection; and can include the use of liposomes, microemulsions or the like, which can facilitate introduction of the polynucleotide into the cell and can protect the polynucleotide from degradation prior to its introduction into the cell. A particularly useful method comprises incorporating the polynucleotide into microbubbles, which can be injected into the circulation. An ultrasound source can be positioned such that ultrasound is transmitted to the tumor, wherein circulating microbubbles containing the polynucleotide are disrupted at the site of the tumor due to the ultrasound, thus providing the polynucleotide at the site of the cancer. The selection of a particular method will depend, for example, on the cell into which the polynucleotide is to be introduced, as well as whether the cell is isolated in culture, or is in a tissue or organ in culture or *in situ*.

[0120] Introduction of a polynucleotide into a cell by infection with a viral vector is particularly advantageous in that it can efficiently introduce the nucleic acid molecule into a cell. Moreover, viruses are very specialized and can be selected as vectors based on an ability to infect and propagate in one or a few specific cell types. Thus, their natural specificity can be used to target the nucleic acid molecule contained in the vector to specific cell types. As such, a vector based on an HIV can be used to infect T cells, a vector based on an adenovirus can be used, for example, to infect respiratory epithelial cells, a vector based on a herpesvirus can be used to infect neuronal cells, and the like. Other vectors, such as adeno-associated viruses can have greater host cell range and, therefore, can be used to infect various cell types, although viral or non-viral vectors also can be modified with

specific receptors or ligands to alter target specificity through receptor mediated events. A polynucleotide of the invention, or a vector containing the polynucleotide can be contained in a cell, for example, a host cell, which allows propagation of a vector containing the polynucleotide, or a helper cell, which allows packaging of a viral vector containing the polynucleotide. The polynucleotide can be transiently contained in the cell, or can be stably maintained due, for example, to integration into the cell genome.

[0121] A method of the invention also can be practiced by directly providing desired polypeptide to the site of a cell exhibiting unregulated growth in the subject. The polypeptide can be produced and isolated, and formulated as desired, using methods as disclosed herein, and can be contacted with the cell such that the polypeptide can cross the cell membrane of the target cells. Where the desired polypeptide is contacted with a cell in an organism, it can comprise a fusion protein, which includes a peptide or polypeptide component that facilitates transport across the cell membrane, for example, a human immunodeficiency virus (HIV) TAT protein transduction domain, and can further comprise a nuclear localization domain operatively linked thereto. Alternatively, or in addition, the polypeptide can be formulated in a matrix that facilitates entry of the polypeptide into a cell.

[0122] For administration to a living subject, an agent such as a demethylating agent, a polynucleotide, or a polypeptide useful for practicing a therapeutic method of the invention generally is formulated in a composition suitable for administration to the subject. Thus, the invention provides compositions containing an agent that is useful for restoring regulated growth to a cell exhibiting unregulated growth due to methylation silenced transcription of one or more genes. As such, the agents are useful as medicaments for treating a subject suffering from a pathological condition associated with such unregulated growth.

[0123] Such compositions generally include a carrier that can be acceptable for formulating and administering the agent to a subject. Such acceptable carriers are well known in the art and include, for example, aqueous solutions such as water or physiologically buffered saline or other solvents or vehicles such as glycols, glycerol, oils such as olive oil or injectable organic esters. An acceptable carrier can contain

physiologically acceptable compounds that act, for example, to stabilize or to increase the absorption of the conjugate. Such physiologically acceptable compounds include, for example, carbohydrates, such as glucose, sucrose or dextrans, antioxidants, such as ascorbic acid or glutathione, chelating agents, low molecular weight proteins or other stabilizers or excipients. One skilled in the art would know that the choice of an acceptable carrier, including a physiologically acceptable compound, depends, for example, on the physico-chemical characteristics of the therapeutic agent and on the route of administration of the composition, which can be, for example, orally or parenterally such as intravenously, and by injection, intubation, or other such method known in the art. The pharmaceutical composition also can contain a second reagent such as a diagnostic reagent, nutritional substance, toxin, or therapeutic agent, for example, a cancer chemotherapeutic agent.

[0124] The agent can be incorporated within an encapsulating material such as into an oil-in-water emulsion, a microemulsion, micelle, mixed micelle, liposome, microsphere, microbubbles or other polymer matrix (see, for example, Gregoriadis, *Liposome Technology*, Vol. 1 (CRC Press, Boca Raton, FL 1984); Fraley, et al., *Trends Biochem. Sci.*, 6:77 (1981), each of which is incorporated herein by reference). Liposomes, for example, which consist of phospholipids or other lipids, are nontoxic, physiologically acceptable and metabolizable carriers that are relatively simple to make and administer. "Stealth" liposomes (see, for example, U.S. Patent Nos. 5,882,679; 5,395,619; and 5,225,212, each of which is incorporated herein by reference) are an example of such encapsulating materials particularly useful for preparing a composition useful in a method of the invention, and other "masked" liposomes similarly can be used, such liposomes extending the time that the therapeutic agent remain in the circulation. Cationic liposomes, for example, also can be modified with specific receptors or ligands (Morishita et al., *J. Clin. Invest.*, 91:2580-2585 (1993), which is incorporated herein by reference). In addition, a polynucleotide agent can be introduced into a cell using, for example, adenovirus-polylysine DNA complexes (see, for example, Michael et al., *J. Biol. Chem.* 268:6866-6869 (1993), which is incorporated herein by reference).

[0125] The route of administration of the composition containing the therapeutic agent will depend, in part, on the chemical structure of the molecule. Polypeptides and

polynucleotides, for example, are not particularly useful when administered orally because they can be degraded in the digestive tract. However, methods for chemically modifying polypeptides, for example, to render them less susceptible to degradation by endogenous proteases or more absorbable through the alimentary tract are disclosed herein or otherwise known in the art (see, for example, Blondelle et al., *supra*, 1995; Ecker and Crook, *supra*, 1995). In addition, a polypeptide agent can be prepared using D-amino acids, or can contain one or more domains based on peptidomimetics, which are organic molecules that mimic the structure of a domain; or based on a peptoid such as a vinylogous peptoid.

[0126] A composition as disclosed herein can be administered to an individual by various routes including, for example, orally or parenterally, such as intravenously, intramuscularly, subcutaneously, intraorbitally, intracapsularly, intraperitoneally, intrarectally, intracisternally or by passive or facilitated absorption through the skin using, for example, a skin patch or transdermal iontophoresis, respectively. Furthermore, the composition can be administered by injection, intubation, orally or topically, the latter of which can be passive, for example, by direct application of an ointment, or active, for example, using a nasal spray or inhalant, in which case one component of the composition is an appropriate propellant. A pharmaceutical composition also can be administered to the site of a pathologic condition, for example, intravenously or intra-arterially into a blood vessel supplying a tumor.

[0127] The total amount of an agent to be administered in practicing a method of the invention can be administered to a subject as a single dose, either as a bolus or by infusion over a relatively short period of time, or can be administered using a fractionated treatment protocol, in which multiple doses are administered over a prolonged period of time. One skilled in the art would know that the amount of the composition to treat a pathologic condition in a subject depends on many factors including the age and general health of the subject as well as the route of administration and the number of treatments to be administered. In view of these factors, the skilled artisan would adjust the particular dose as necessary. In general, the formulation of the composition and the routes and frequency of administration are determined, initially, using Phase I and Phase II clinical trials.

[0128] The composition can be formulated for oral formulation, such as a tablet, or a solution or suspension form; or can comprise an admixture with an organic or inorganic carrier or excipient suitable for enteral or parenteral applications, and can be compounded, for example, with the usual non-toxic, pharmaceutically acceptable carriers for tablets, pellets, capsules, suppositories, solutions, emulsions, suspensions, or other form suitable for use. The carriers, in addition to those disclosed above, can include glucose, lactose, mannose, gum acacia, gelatin, mannitol, starch paste, magnesium trisilicate, talc, corn starch, keratin, colloidal silica, potato starch, urea, medium chain length triglycerides, dextrans, and other carriers suitable for use in manufacturing preparations, in solid, semisolid, or liquid form. In addition auxiliary, stabilizing, thickening or coloring agents and perfumes can be used, for example a stabilizing dry agent such as triulose (see, for example, U.S. Patent No. 5,314,695).

[0129] The following examples are intended to illustrate but not limit the invention.

#### **EXAMPLE 1**

##### **IDENTIFICATION OF EPIGENETIC SILENCED TUMOR SUPPRESSOR GENES IN ESOPHAGEAL CANCER CELLS**

[0130] This example provides a genomic screening method for identifying epigenetically silenced genes, including epigenetically silenced tumor suppressor genes, associated with esophageal squamous cell carcinoma cells (see, also, Yamashita et al., *Cancer Cell* 2:485-495, 2002, which is incorporated herein by reference).

#### **METHODS**

##### **Cell lines and tissue samples**

[0131] Esophageal squamous cell carcinoma (ESCC) cell lines TE1, TE2, TE3, TE4, TE5, TE7, TE13, KYSE30, KYSE70, KYSE110, KYSE140, KYSE150, KYSE200, KYSE410 and KYSE520 were obtained from the Cell Response Center for Biomedical Research Institute of Department, Aging and Cancer, Tohoku University (TE series) and kindly provided by Dr. Shimada in the Department of Surgery, Kyoto University (KYSE series). Cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum for isolation of DNA and RNA. Primary ESCC tumors and corresponding adjacent normal

tissues were obtained from the Gastroenterology Division, the Department of Medicine, and the University of Maryland School of Medicine.

**5-Aza-2'-deoxycytidine (5Aza-dC) and Trichostatin A (TSA) treatment of cells**

[0132] Cells were split to low density ( $5 \times 10^5$  per T-25 flask) 12 to 24 hr before treatment, then treated for 3, 4, or 5 days with 1  $\mu$ M or 5  $\mu$ M 5Aza-dC (Sigma) from 100-mM 50% acetic acid dissolved stock or were mock-treated with the same volume of phosphate buffered saline (PBS) including the same acetic acid. Following an initial incubation (48 hr) of 5Aza-dC, a final concentration of 300 nM TSA (Sigma) was added to the media from a 5 mM ethanol dissolved stock, or cells were mock-treated with an identical volume of ethanol.

**Microarray and RT-PCR analysis**

[0133] Oligonucleotide microarray analysis was performed using the GeneChip<sup>TM</sup> Human Genome U95Av2 Array (Affymetrix), which contains 12,599 genes, according to the manufacturer's instruction. Genes that were up-regulated by pharmacologic treatment were identified according to the manufacturer's algorithm. RNA was isolated using TRIZOL reagent (Invitrogen Corp.) and reverse-transcribed total RNA (8  $\mu$ g) with M-MLV (Invitrogen Corp.); one one-hundredth of the cDNA product was used as a template for PCR. RT-PCR was performed at 24 to 30 cycles: 95°C for 1 min, 54 or 56°C for 1 min, and 72°C for 1 min. Exemplary amplification primer pairs for RT-PCR are shown in Table 3 (SEQ ID NOS:7 to 64). In control reactions, no amplification of PCR products was seen without reverse transcription (-RT).

[0134] Preliminary analysis using a 2-fold increase as a cut-off yielded many additional genes. However, few of these genes were up-regulated in more than one cell line and several genes such as BIN1, BRCA-associated protein 1 (BAP-1), and MAP kinase 8 interacting protein 3 (JNK proteins scaffolding protein), harbored no methylation in the promoter region. Such genes could be on a pathway regulated by more upstream genes epigenetically regulated in the cell lines examined. In comparison, a cut-off at a 3-fold increase of expression routinely identified epigenetically silenced genes with promoter hypermethylation. Accordingly, a 3-fold increase was used in the studies disclosed herein.

**Sequencing analysis**

[0135] Genomic DNA was extracted from TRIZOL reagent and bisulfite modification of genomic DNA was performed as described (Merlo et al., *Nature Med.* 1:686-692, 1995, which is incorporated herein by reference). Bisulfite-treated DNA was amplified for the 5' region that included the ATG start sites or proposed transcriptional start sites (approx. 200 to 500 bp) using primer sets made for the 25 genes (22 listed genes, and MAPK8IP3, BIN1, and BAP-1). All the PCR products were gel-extracted (Qiagen) and applied to an Applied Biosystems 3700 DNA analyzer using BD terminator dye (Applied Biosystems) and nested primers or forward primers (see Table 4; F2 primers).

**Methylation-specific PCR**

[0136] Bisulfite-treated DNA was amplified with either a methylation-specific or unmethylation-specific primer set for Apolipoprotein D at 33 cycles: 96°C for 30 sec, 59°C (methylated) and 55°C (unmethylated) for 30 sec, and 72°C for 30 sec. Methylation-specific primer sequences for apolipoprotein D were designed using 5'-CACACGCGCGAAAACAATAT-3' (SEQ ID NO:1) as the forward primer, and 5'-TATGTATGTTACGTTTCGTCG-3' (SEQ ID NO:2) as the reverse primer. Unmethylation-specific primer sequences were 5'-CACACAAAAACAATATCTCATTCT-3' (SEQ ID NO:3) and 5'-TTTTTTATGTATGTTATGTTTGTTG-3' (SEQ ID NO:4). In other experiments, the methylation specific primers set forth as SEQ ID NOS:82 and 84 were used (see Table 4). Additional methylation specific primers are shown in Table 4 (SEQ ID NOS:65 to 127), wherein the forward primer is indicated as F1 or F2, and the reverse primer is indicated as R; and wherein the "PCR amplification" column indicates the methylation specific primer pair used to obtain the results disclosed herein, and the "sequence" column indicates primers used for bisulfite sequencing.

**Construction of human expression vectors**

[0137] A full length CRIP1 cDNA was isolated from TE2 cells using PCR with the primer sets 5'-CAGAAGCTTCCACCATGCCCAAGTGCCCAAGTGC-3' (SEQ ID NO:5) and 5'-CTCTCGGTGTGAAAGTTCATTAGATCTGAC-3' (SEQ ID NO:6), which include Hind III and Xba I recognition sites. The PCR product was cut from a gel, cleaved

with Hind III and Xba I, and ligated to a Hind III-Xba I digested pcDNA3<sup>TM</sup> vector (Invitrogen), which harbors a CMV promoter. One clone, pcDNA3-CRIP1, harbored an insertion with a sense orientation and a correct sequence. p53 was amplified as a template of pRCC-p53 (Osada et al., *Nature Med.* 4:839-843, 1998, which is incorporated herein by reference), subcloned into the Hind III and Xba I sites of a pcDNA3<sup>TM</sup> vector, and sequenced. An Apo D cDNA inserted into pcDNA3<sup>TM</sup> vector and STAT3C cDNA inserted into pcDNA3<sup>TM</sup> vector also were utilized (see, e.g., Bromberg et al., *Cell* 98:295-303, 1999).

#### **Transfection and colony formation assay**

[0138] Colony formation assays were performed in monolayer culture (Yoshikawa et al., *supra*, 2001). Cells were plated at  $2 \times 10^4$  cells per well using 6-well plates, and transfected with 1  $\mu$ g of either pcDNA3-p53, pcDNA3-STAT3C, pcDNA3-CRIP1, pcDNA3-ApoD, or pcDNA3-mock (no insert) using Lipofectamine Plus<sup>TM</sup> transfection reagents (Invitrogen) according to the manufacturer's protocol. The cells were then detached and plated on 100 mm tissue culture dishes at 24 to 48 hr post-transfection, and simultaneously harvested at 48 hr after transfection to confirm their expression at the mRNA level (RT-PCR) for ApoD. Cells transfected with CRIP1 died rapidly by 48 hr after transfection; thus mRNA levels were evaluated 24 hr after transfection. ApoD and CRIP-1 RNA levels in the cells were 1.5-2.0 fold higher than basal expression of unmethylated cell lines. Cells were selected with G418 (1 mg/ml), and colonies were counted 2 weeks after transfection. For treatment with Neuromedin U (NU; -Phoenix Pharmaceutical), the colony focus assay included 100  $\mu$ M NU in control PBS or the medium.

## **RESULTS**

### **Pharmacologic unmasking of transcriptionally repressed genes**

[0139] Treatment of cells with the demethylating agent 5aza-dC (1  $\mu$ M or 5  $\mu$ M) for 3 to 5 days, and with or without the histone deacetylase inhibitor, TSA (300 nM; last 24 hours), was used to reactivate genes epigenetically silenced in 3 ESCC cell lines. Following treatment, changes in gene expression were measured using microarray chips containing 12599 transcripts (Affymetrix); randomly picked genes were confirmed by RT-PCR. As expected, treatment with these agents resulted in up-regulation (i.e., at least a 3.0 fold

increase) of more than 500 unique genes (Table 1). Almost all of the genes (>80%) were included in the 5 $\mu$ M 5Aza-dC treatment group, but a few genes were identified at a lower 5Aza-dC concentration or by addition of TSA (see below). It was reasoned that commonly reactivated genes, inactivated in 2 or 3 ESCC cell lines were more likely to represent frequently inactivated tumor suppressor genes (TSGs; 120 genes). The number of candidate genes was further diminished by comparing expression patterns (see world wide web, at URL "cgap.nci.nih.gov") in normal esophagus and carcinoma tissue samples (eliminating those genes not expressed in normal tissue), and excluding unknown genes. A more detailed analysis was performed on 58 genes that were commonly up-regulated after demethylation treatments (Figure 1; Table 2).

[0140] Fifty-three of the 58 genes (91%) harbored CpG sites and 44 genes (76%) harbored dense CpG islands (GC content>60% or CpG content>15%) in the promoter region (Table 2; CpG lane). RT-PCR was performed to confirm up-regulation after treatment in 25 randomly selected genes in the 3 key ESCC cell lines. All 25 genes demonstrated robust reexpression after 5  $\mu$ M demethylation treatment (Table 2; ESCC cell line/unmasking). Genes such as cytokine-like factor-1 (CLF-1) and Hep27 demonstrated synergistic reactivation with 1  $\mu$ M 5Aza-dC and 300 nM TSA as compared to TSA or 5Aza-dC alone in several cancer cells.

#### **Expression and promoter hypermethylation in ESCC cell lines**

[0141] A subset of the 58 genes was examined for silencing or down-regulation in an additional 12 ESCC cell lines. TE4 and TE5 retained expression of apolipoprotein D, while the remaining cell lines were down-regulated at the mRNA level when compared to normal esophagus. Neuromedin U was completely silenced in 6 cell lines, and other cell lines also showed a marked reduction in expression as compared with normal esophagus. Cystein rich intestinal protein 1 (CRIP1) was completely silenced in 8 out of 15 ESCC cell lines (53%), while normal esophageal tissues showed abundant expression of CRIP1. Several other selected candidate genes, including cytokine-like factor-1 (CLF-1), swiprosin-2, CRBP, metallothionein 1G, keratin 14, crystallin alpha 2, and IL-1 receptor 2, demonstrated considerable down-regulation in ESCC cell lines.

[0142] In order to confirm promoter hypermethylation in reactivated genes, the promoter regions of 22 genes (21 genes harbored dense CpG islands plus Apolipoprotein D) were examined using bisulfite sequencing (Table 2). Most of the candidate genes (*Tbc1d1*, lysosomal neuraminidase precursor, Apolipoprotein J, KLF6, putative cyclin G1 interacting protein, and XAP-5) with moderate basal expression before treatment by RT-PCR were invariably unmethylated (lysosomal neuraminidase precursor, putative cyclin G1 interacting protein). On the other hand, 13 genes ( $\alpha$ -tubulin, swiprosin-2, insulin-like growth factor binding protein 2 (IGFBP2), cellular retinol-binding protein (CRBP), apolipoprotein D (ApoD), neuromedin U (NU), claudin-3, uncoupling protein-2 (UCP-2), cysteine-rich intestinal protein 1 (CRIP1), metallothionein 1G (MT 1G), apolipoprotein C1 (Apo C1), cytokine-like factor-1 (CLF-1), transglutaminase-2) retained high cytosine content in their CpG islands or CpG sites deemed critical for transcription (APOD) after bisulfite treatment, indicating heavy cytosine methylation (see Table 2; "CpG<sup>d</sup> lane - "CpG status" - genes designated "M"). In all of these genes except swiprosin-2, methylation status correlated tightly with expression status. For example, KYSE30 was silenced for NU expression and harbored dense methylation of the promoter, while KYSE410 and KYSE520 both expressed NU and were free of promoter methylation. For ApoD, all ESCC cell lines except TE5 demonstrated methylation of the promoter, while TE5 cells, which expressed ApoD abundantly, did not harbor any methylation. TE2, TE4, and TE13 cells showed weak expression of Apo D mRNA and approximately 50% of the cells harbored unmethylated alleles by direct sequence of bisulfite treated DNA.

#### **Expression and promoter hypermethylation in primary ESCC tumors**

[0143] The 13 methylated genes (Table 2; "cpg<sup>d</sup>" lane, genes designated "M") were examined for promoter methylation in ESCC tissues by (MSP) or direct sequence analysis. Ten of the 13 genes harbored tumor specific promoter methylation (Figure 2). The frequency of tumor methylation ranged from 10% (UCCP-2) to 80% (apolipoprotein C1). ApoD was methylated in almost all primary ESCC tissues (80%), and showed low-level methylation in some normal tissues by MSP. Swiprosin-2 and  $\alpha$  tubulin showed methylation in normal esophageal mucosa specimens suggesting tissue-specific, but not tumor specific hypermethylation. None of the 10 primary tumors tested harbored IGFBP-2 methylation.

[0144] Expression of the 13 genes was then examined in 5 primary ESCC tissues; reduced expression was detected in the primary cancers as compared to the corresponding normal tissues (Table 2). NU and ApoD were markedly repressed at the mRNA level in several primary tumors as compared to the corresponding normal tissue. Except for swisprosin-2, every gene that was methylated in primary tumors demonstrated a marked decrease in expression at the RNA level. For swisprosin-2, methylation did not correlate with decreased expression in primary tumors or cell lines (Table 2). A few tumors did not harbor methylation, but still demonstrated occasional down-regulation of a particular gene (Table 2), presumably due to other mechanisms.

#### **Tumor suppressor activity**

[0145] Three of the methylated genes (CRIP, ApoD, and NU) were examined for the ability to function as suppressors of tumor growth in KYSE30, which was silenced for all 3 genes. The 3 genes were examined using a colony focus assay with G418 selection after transfection with each gene or control vector (pcDNA3<sup>TM</sup> vector). STAT3 is an oncogenic protein, and STAT3C is a constitutive active form (Bromberg et al., *supra*, 1999). Since STAT3 expression did not change after 5Aza-dC treatments, it was used as a negative control of tumor suppressive activity. In the case of NU, the actual protein was used at a concentration of 100  $\mu$ M. All 3 genes demonstrated potent tumor suppressing activity, with a marked reduction of colony forming ability observed following transfection (ApoD, 61.3 $\pm$ 15.0%; CRIP1, 18.3 $\pm$ 15.7%) or addition of protein (NU 33.6 $\pm$ 12.3%) in 3 independent experiments (see, for example, Figure 3). To further confirm a role for CRIP1 in expression, IRES-CRIP1 was transiently transfected into KYSE30 cells. Cells expressing high GFP protein (and thus CRIP1 simultaneously) showed classic morphological changes of apoptosis, including rounding, apoptotic body appearance, and nuclear shrinkage. A TUNEL assay confirmed DNA fragmentation in CRIP1 expressing cells.

[0146] Pharmacologic reversal of epigenetic silencing uncovered a myriad of transcriptionally repressed genes in ESCC. Several unknown TSG candidates were identified using a cut-off of a 3 fold-increase by microarray analysis and an intuitive algorithm. The majority of genes were identified by high dose 5aza-dC treatment, and a

subset of genes was reactivated by synergistic treatment with low dose 5aza-dC and TSA, as has been demonstrated in colon cancer cell lines (Cameron et al., *supra*, 1999; Suzuki et al., *supra*, 2002). This survey may represent a minimal number of up-regulated genes, as reversal of epigenetic silencing is likely to occur in a subset of cells with variable re-expression (Cameron et al., *supra*, 1999). A more complex approach involving selective cloning identified many methylated targets in colorectal cancer and can be used to identify more subtle targets (Suzuki et al., *supra*, 2002). The use of high dose 5Aza-dC likely induced re-expression in a higher number of cells, thus facilitating the direct hybridization approach used for the present studies. Multiple approaches to reverse epigenetic silencing are likely to yield the most comprehensive gene surveys. In addition, other algorithms and better demethylation or HDAC inhibition can improve the yield by further unmasking of methylated targets.

[0147] Neuromedin U (NU) is proposed to be involved in normal esophageal mucosa integrity, and NU as well as the recently identified cognate receptor (Hedrick et al., *Mol. Pharmacol.* 58:870-875, 2000) are abundantly expressed in normal esophageal mucosa (Hedrick et al., *supra*, 2000; Lynch et al., *Nature* 406:70-74, 2000). The NU receptor (FM3) is a G protein coupled receptor that can signal through PI3 kinase gamma, recently confirmed to block growth of human colon cancer cells (Sasaki et al., *Nature* 406:897-902, 2000). Moreover, in the present study, the list of candidate TSGs includes neuromedin B and RSG2, which are involved in the same pathway.

[0148] CRIP has a LIM domain, which is involved in carcinogenesis (e.g. Lmo2 and Lmo4). Lmo2 is a transcriptional factor proposed to play an oncogenic role in T cell leukemogenesis (Grutz et al., *EMBO J.* 17:4594-4605, 1998), perhaps by enhancing angiogenesis (Yamada et al., *Oncogene* 21:1309-1315, 2002). Lmo4 is over-expressed in breast cancer cells and binds with BRCA1 resulting in suppression of BRCA1 transcriptional activity (Visvander et al., *Proc. Natl. Acad. Sci. USA* 98:14452-14457, 2001; Sum et al., *J. Biol. Chem.* 277:7849-7856, 2002). Paxillin is a focal adhesion-associated adapter protein with multiple LIM domains involved in cell spreading and motility (Schaller, *Oncogene* 20:6459-6472, 2001). Paxillin LIM binds with alpha-tubulin (Herrerros et al., 2000), which is a partner for other critical tumor suppressors including APC and Fhit.

The LIM gene identified herein (CRIP) is a very small molecule (open reading frame of 273 bp) that can modulate other LIM proteins in a dominant negative manner and potentially modulate or affect many pathways in carcinogenesis. Moreover, a yeast two hybrid screen identified Ubc13 as a binding partner, implicating CRIP in NF-kappa B and JNK pathways, which are critical for apoptosis (Wang et al., *Nature* 412:346-351, 2001).

[0149] Apolipoprotein D is associated with cell growth arrest (Do Carmo et al., *J. Biol. Chem.* 277:5514-5523, 2002), but its underlying mechanism is unknown. Other apolipoproteins such as apolipoprotein CI and apolipoprotein J, are included among the 58 genes identified herein. Apolipoprotein J possesses a potent ability to induce cell death (Han et al., *Nature Med.* 7:338-343, 2001). Apolipoprotein D binds to a cytokine type receptor that mediates MAP kinase signaling (Liu et al., *FASEB J.* 15:1329-1331, 2001). This cytokine signaling pathway also can be used by recognized tumor suppressors like SOCS-1 (Yoshikawa et al., *supra*, 2001) and, as indicated by the present results, the IL-1 receptor antagonist (IL-1 R2) (Colotta et al., *Science* 261:472-475, 1993) and CLF-1 (Elson et al., *J. Immunol.* 161:1371-1379, 1998) also can be involved in this pathway.

[0150] The chromosomal localization of the 58 candidate genes is listed in Table 2. Many TSG candidates were clustered in specific chromosomal regions (Table 2), suggesting the presence of methylated chromosomal regions with highly dense methylation and reduced gene expression in tumors, which can be unmasked after pharmacological demethylation treatment. Apolipoprotein D, NU, and CRIP1 are localized at 3q26, 4q12, and 14q24, respectively. All of these loci harbor chromosomal deletions or LOH in various cancers, and 3q26 also is a fragile site. The present study revealed that ten genes were methylated in a tumor specific pattern but, whether methylated or not, all of these genes remain candidates in TSG pathways. For example, MUC2 was one of the genes identified in the present study; MUC2 knockout mice are predisposed to tumor formation (Velcich et al., *Science* 295:1726-1729 2002). Thus, many of the genes identified in the present study can represent TSG candidates that have not yet been examined for genetic or epigenetic inactivation in cancers.

[0151] Candidate genes and pathways epigenetically regulated in ESCC were identified using functional reversal of methylation and deacetylation followed by hybridization on microarrays. The results disclosed herein demonstrate that this approach is rapid and robust, and it can easily be repeated in other cancer cell lines to comprehensively search for epigenetically silenced suppressor genes. Complementary genomic array approaches that search for methylated CpG islands can be compared to functional reactivation surveys (Gitan et al., *Genome Res.* 12:158-164, 2002; Adorjan et al., *Nucleic Acid Res.* 30:e21, 2002; Shi et al., *Cancer Res.* 62:3214-3220, 2002). Genes that harbor CpG islands in the promoter regions and methylation in tumor tissue are likely to be bonafide TSGs (Table 2). The present approach thus yielded 3 new TSGs and many more remain to be examined.

[0152] The use of MSP allows rapid detection of the frequency of inactivation in primary tumors by methylation while functional analysis allows rapid assessment of suppressor activity. The identified genes can provide a means to identify and manipulate the biologic progression of ESCC and, therefore, represent important therapeutic targets. Moreover, the rapid development of MSP assays after gene identification allows robust analysis of primary tumors and other clinical samples for implementation of promising molecular detection approaches (see, for example, Esteller et al., *supra*, 1999; Kawakami et al., *J. Natl. Cancer Inst.* 92:1805-1811, 2000; Jeronimo et al., *J. Natl. Cancer Inst.* 93:1747-1752, 2001).

## EXAMPLE 2

### IDENTIFICATION OF EPIGENETIC SILENCED TUMOR SUPPRESSOR GENES IN HEAD AND NECK CANCER CELLS

[0153] This example extends the results disclosed above for esophageal cancer cells to head and neck squamous cell carcinoma (HNSCC) cells.

[0154] HNSCC cells were incubated with 10  $\mu$ M 5Aza-dC or with 0.1  $\mu$ M 5Aza-dC and 300 nM TSA and screened as described in Example 1. Reactivation of epigenetically silenced genes was observed for both groups of treated cells. Exemplary genes showing at least a 2-fold increase in expression are shown in Tables 5 and 6 (fold increase shown in second column).

[0155] These results demonstrate that the genomic screening method disclosed in Example 1 can be extended to other cancer cell types. Further analysis of the re-expressed genes in HNSCC cells as disclosed in Example 1 can identify those genes having tumor suppressor activity.

[0156] Although the invention has been described with reference to the above example, it will be understood that modifications and variations are encompassed within the spirit and scope of the invention. Accordingly, the invention is limited only by the claims, which follow Tables 1 to 6.

Table 1. Genes upregulated by microarray analysis after treatments

|                                | KYSE30 | KYSE410 | KYSE520 |
|--------------------------------|--------|---------|---------|
| 1 $\mu$ M 5Aza-dC              | 46     | 15      | 5       |
| 1 $\mu$ M 5Aza-dC + 300 nM TSA | 57     | 21      | 51      |
| 5 $\mu$ M 5Aza-dC              | 242    | 334     | 149     |
| Unique gene number             | 289    | 363     | 185     |
| Total unique = 565 genes       |        |         |         |

Table 2. Identification of candidates of tumor suppressor genes in esophageal carcinoma

| Gene bank | Gene Name                                 | ESCC cell line |                                         |                         |                                                           | known or proposed function |
|-----------|-------------------------------------------|----------------|-----------------------------------------|-------------------------|-----------------------------------------------------------|----------------------------|
|           |                                           | Chr.           | CpG <sup>a</sup> Unmasking <sup>b</sup> | expression <sup>c</sup> | CpG <sup>d</sup> expression <sup>e</sup> CpG <sup>f</sup> |                            |
| M21302    | Small proline rich protein (sprl)         | 1              | (-)                                     | •                       | •                                                         | UV-induced gene            |
| A1813532  | TNF receptor 18                           | 1p36           | (++)                                    | •                       | •                                                         | cytokine receptor          |
| A1885852  | Histone 2A.2                              | 1q21           | (++)                                    | •                       | •                                                         | nucleosome protein         |
| J05581    | MUC1                                      | 1q21           | (++)                                    | Yes                     | U                                                         | tumor antigen              |
| L13463    | Regulator of G protein signaling 2 (RGS2) | 1q31           | (++)                                    | •                       | •                                                         | G-protein signal           |
| AF010309  | Pig3                                      | 2p23           | (+)                                     | •                       | •                                                         | p53-induced gene           |
| X06956    | alpha-tubulin (b alpha 1)                 | 2p36           | (++)                                    | •                       | M                                                         | microtubule                |
| X59770    | IL-1 R2                                   | 2q12           | (-)                                     | Yes                     | •                                                         | cytokine receptor          |
| A8011103  | KIF5C                                     | 2q23           | (?)                                     | Yes                     | •                                                         | cytoplasmic transport      |
| X63368    | HSP40 homolog (HSJ1)                      | 2q32           | (+)                                     | •                       | •                                                         | stress-induced gene        |
| W27472    | Swi/brasin-2                              | 2q36           | (++)                                    | Yes                     | M                                                         | growth arrest?             |
| S37730    | IGFBP2                                    | 2q36           | (++)                                    | •                       | M                                                         | IGF signal                 |
| M11433    | Cellular retinol-binding protein (CRBP)   | 3q21           | (++)                                    | •                       | M                                                         | retinol-binding protein    |
| J02611    | Apolipoprotein D                          | 3q26           | (+)                                     | Yes                     | M                                                         | growth arrest              |
| AB029031  | Tbc1d1                                    | 4p14           | (++)                                    | Yes                     | M                                                         | oncosis                    |
| AF084481  | Transmembrane protein (WFS1)              | 4p16           | (++)                                    | •                       | •                                                         | Wolfram syndrome           |
| X76029    | Neuramedin U                              | 4q12           | (++)                                    | Yes                     | M                                                         | G-protein receptor ligand  |
| X57522    | TAP1                                      | 6p21           | (++)                                    | No                      | U                                                         | tumor antigen processing   |
| AF040958  | Lysosomal neuraminidase precursor         | 6p21           | (++)                                    | Yes                     | U                                                         | enzyme                     |
| X70683    | Sry                                       | 6p22           | (++)                                    | •                       | •                                                         | sex-determining gene       |
| AB000714  | Claudin-3                                 | 7q11           | (++)                                    | •                       | M                                                         | tight junction             |
| M25915    | Apolipoprotein J                          | 8p21           | (++)                                    | Yes                     | M                                                         | apoptosis                  |
| M22488    | Bone morphogenic protein 1 (BMP-1)        | 8p21           | (++)                                    | •                       | U                                                         | TGF-beta superfamily       |
| AF001461  | KLF6 (Zf9)                                | 10p15          | (++)                                    | Yes                     | •                                                         | tumor suppressor gene      |
| M13755    | Interferon-induced 17-kDa/15-kDa          | 11p36          | (++)                                    | •                       | •                                                         | Interferon-induced gene    |
| U94592    | Uncoupling protein 2                      | 11q13          | (++)                                    | Yes                     | M                                                         | apoptosis                  |
| W68521    | Cystatin E/M (6)                          | 11q13          | (++)                                    | •                       | •                                                         | protease inhibitor         |
| AF001436  | Cdc42 effector protein 2 (CEP2)           | 11q13          | (++)                                    | •                       | •                                                         | motility inhibition        |
| X15675    | pTR7                                      | 11q13          | (++)                                    | •                       | •                                                         | repetitive sequence gene   |

Table 2 Continued

|           |                                               |            |     |   |                           |
|-----------|-----------------------------------------------|------------|-----|---|---------------------------|
| AI038340  | Crystallin alpha 8                            | 11q22 (++) | Yes | • | stress-induced gene       |
| AJ001684  | NKG2C                                         | 12p13 (-)  | •   | • | NK cell recognition       |
| AJ001685  | NKG2E                                         | 12p13 (-)  | Yes | • | NK cell recognition       |
| M20681    | GLUT3                                         | 12p13 (+)  | •   | • | glucose transport         |
| U31875    | Hep27                                         | 14q11 (+)  | Yes | • | growth arrest?            |
| X81637    | Clahtin light chain b                         | 14q21 (++) | •   | • | membrane recycling        |
| X67325    | Interferon stimulatory gene 12 (p27)          | 14q32 (+)  | Yes | • | IFN-induced gene          |
| AI017574  | Cystein rich protein with LIM (CRIP1)         | 14q32 (++) | Yes | • | LIM protein               |
| AI985272  | Neuromedin B                                  | 15q22 (++) | •   | • | G-protein receptor ligand |
| NM_002201 | HEM45 (Interferon stimulatory gene 20)        | 15q26 (++) | Yes | • | IFN-induced gene          |
| J03910    | Metallothionein-1G                            | 16q (++)   | •   | M | apoptosis-related gene    |
| NM_004165 | Rad                                           | 16q22 (++) | •   | U | motility inhibition       |
| AA976838  | Apolipoprotein C1                             | 16q22 (++) | Yes | • | growth promotion?         |
| J00124    | Keratin 14                                    | 17q12 (+)  | Yes | • | intermediate filament     |
| U20982    | IGFBP4                                        | 17q12 (++) | Yes | • | IGF signal                |
| Z19574    | Cytokeratin 17                                | 17q12 (++) | •   | • | intermediate filament     |
| AF059293  | Cytokine-like factor-1 (CLF-1)                | 19p13 (++) | Yes | • | cytokine receptor homolog |
| D12620    | Cytochrome P-450L1BV                          | 19p13 (+)  | •   | • | electron transmission     |
| AF061812  | Keratin 16                                    | 19q12 (++) | •   | • | intermediate filament     |
| U76702    | Follistatin-related protein FLRG              | 19q13 (++) | Yes | • | TGF-beta related gene     |
| AF044968  | Nectin 2                                      | 19q13 (++) | •   | U | tight junction            |
| AA1524 06 | "Cytochrome oxidase c, type VIIa"             | 19q13 (++) | •   | • | electron transmission     |
| M37457    | "Na+, K+-ATPase catalytic subunit, alpha-III" | 19q13 (++) | •   | • | TGF-beta related gene     |
| U61837    | Pulative cyclin G1 Interacting protein        | 20p13 (++) | Yes | • | ion transport             |
| M55153    | Transglutaminase 2 (tissue glutaminase)       | 20q11 (++) | Yes | U | cell cycle (G2/M)         |
| W72816    | Cleavage stimulation factor                   | 20q13 (++) | •   | M | apoptosis                 |
| X75315    | Seb48 (RPM type RNA binding protein)          | 20q13 (++) | •   | U | BRCA1-related gene        |
| X91817    | Transketolase 2                               | Xq28 (+)   | •   | • | RNA binding protein       |
| AD001530  | XAP-5                                         | Xq28 (++)  | Yes | • | metabolic cofactor        |
|           |                                               |            | No  | • | repellive sequence gene   |

<sup>a</sup>CpG sites, (++) dense CpG islands, (+) CpG sites, (-) no CpG sites in the promoter region.

<sup>b</sup>RT-PCR results of the experiment after treatments. \*: not done. Yes: confirmed silencing in three key cells and reactivation after treatments.

<sup>c</sup>Gene expression by RT-PCR in extended panel of ESCC cells. \*: not done. Yes: silencing confirmed in several cell lines. No: ubiquitously expressed in all investigated cell lines.

<sup>d</sup>CpG status of ESCC cells. M: methylated, U: unmethylated. \*: not done.

<sup>e</sup>Gene expression by RT-PCR in ESCC tissues. Silenced tumors/tumors tested (% silenced).

<sup>f</sup>Promoter methylation by direct sequence in ESCC tissues. Methylated tumors/tumors tested (% in methylation).

Table 3

| RT-PCR primers                    | F                          | R                          |
|-----------------------------------|----------------------------|----------------------------|
| MUC1                              | GCTGGTGCTGCTGTGTTCT (7)*   | AACCTGAGTGGAGTGGAAATGG (8) |
| IL-1 R2                           | TGAAGGCCAGCAATACAACAT      | TGCGTAGTGTCTGAAAACTCA      |
| KIF5C                             | AGAAACTGCAACTGGAACAGG      | CAGCTGCTTGTGAACCTTTGGT     |
| Swiprosin-2                       | TGTCATCGGCCAGTAAGTTTG      | AGAGGAGCAGAGGCAGGAGAGAC    |
| CRBP                              | AGGCCCTCCTGGATGTCACC       | GGAAGATGCTGAGCAACGAG       |
| Apolipoprotein D                  | GCATTTTCATCTTGGGAAGTGC     | TGCAGGTACAGGAATACACGA      |
| Tbc1d1                            | CGAGAAATGAGCAGCGAGAGA (19) | CTCCGAGTCACTGGACAGGTC (20) |
| Neuromedin U                      | GCTCCAATATTACCTCAAGGA      | TCGTCCACTCTGAATCTCTTC      |
| TAP1                              | CAGAACTCTGTACCAGCCC        | CTGGCTGTTTGCATCCAGG        |
| Lysosomal neuraminidase precursor | TGGCTCAGTCGTCATCAATGC      | TCCGGCCTTTCTCATACAGGA      |
| Apolipoprotein J                  | ACAACCCCTCCCAGGCTAAGC      | TCCGCCACGGTCTCCATAAAT      |
| KLF6                              | ATGTGCAGCATCTTCCAGGAG (29) | CCAATGGGTCGGAGGTAAAC (30)  |
| Uncoupling protein 2              | CTAGCAGGCAGCACACACAGGT     | GTCTACAGGGGAGGCGGATGAC     |
| crystallin alpha B                | GACCAGTTCTTCGGAGAGCAC      | ACAGTGAGGACCCCATCAGAT      |
| NKG2E                             | TCAGGAACCGAACAGGAAATA      | CATGAGGAAGGTAAATGCTG       |
| Hep27                             | AATACTCACCGCAAGGTCTG       | CTAGCACAGGGATGAAACCAG      |
| ISG12 (p27)                       | CAGTGTGGCCAAAGTGGTCAG (35) | CAATGGAGCCAGGATGAACT (40)  |
| CRIP-1                            | CCAAGTGCAACAAGGAGGTGT      | GGGTGGTTGCAGTAGGGTTTG      |
| HEM45                             | GCTCGTTGCAGCCTCGTGAAC      | TCAGCACCCCGCAGGGAGACAC     |
| Metallothionein 1G                | TAGGAGGGTGCTTGGTTTTCC      | TTAGCCACAGCCCCAGATTCC      |
| Rad                               | GTGCGCTCTCGTGAGGTCTCG      | CGTTCGCTCTCCACCATAGTG      |
| Keratin 14                        | TAGTGGCTTTGGGGAGGATA (49)  | GTCCACTGTGGCTGTGAGAAT (50) |
| IGFBP4                            | ACAAAAGACTGCCAAGGACAT      | TATTCTCGATAAAGCCTGTGC      |

Table 3 Continued

|                                        |                            |                            |
|----------------------------------------|----------------------------|----------------------------|
| CLF-1                                  | CCGATGTACTCACGCTGGATA      | CATAGATGCCAAAGGGTTGC       |
| FLRG                                   | TTTC TCAGCCCCCAAGCCTCTA    | GTCCCCACACGCACTCAGACT      |
| putative cyclin G1 interacting protein | CGGATGTCACTGTGCTTGAGG      | ATCGTGCCCGGAAGAACTCCTG     |
| transglutaminase                       | TACGATGCGCCCTTTGTCTTT (59) | AGCGGTGTTGTTGGTGATGTG (60) |
| XAP-5                                  | GGAAGCCCAAGCAGGAGAAGAT     | TGAGCAACCGCACATCGTCAT      |
| GAPDH                                  | GTCAACGGATTGTCGTATT (63)   | AGTCTTCTGGGTGGCAGTGAT (64) |

\*SEQ ID NO: - numbered from 7 to 64, from left to right, top to bottom; representative SEQ ID NO: shown.

Table 4

| methylation primers                    | F1                             | F2                           | R                               | PCR<br>amplification | sequence |
|----------------------------------------|--------------------------------|------------------------------|---------------------------------|----------------------|----------|
| MUC1                                   | AACCTACCCCTCCGCCCTCCC (65)*    | AAACCCCTATACCTACCCCA (66)    | GAGGGGGTAGAATAGATTAG (67)       | F2-R                 | F2       |
| RGS2                                   | ATCTCCTATCATAACTTCTCTAAAC      | TACTCCACACCTACCCCAAC (68)    | TGTTTCGCTTTTGTATTAGGTTT (69)    | F2-R                 | F2       |
| alpha-tubulin                          | TGCTCCACCAAACTAACAAT           | AAATTAATACTAAACCAA           | ATTTTATTTAAAGGTTATAAAGAT        | F1-R                 | F2       |
| Swi5prolin-2                           | TATTCCTCTTCAAAAATCATAATCA      | CCAATCCATCCTCAAAACCCCTC      | GTACGTTAGTTTATTTGTTATGG         | F2-R                 | F2       |
| IGFBP2                                 | ATCTCCTCTTCTTTTATAAAAAA        | ATAATCAAAACCAAAAAATA         | GGTAGGGGTACGGTTTGTGAG           | F1-R                 | F2       |
| CRBP                                   | AACCAACTTTAACTCATACCCCTC (82)  | CCTAAACAAACCTAACCCCTT        | TGATGGAGTTAGTTGGTTATAAGT        | F1-R                 | F2       |
| Apolipoprotein D                       | CCCAATGCCAATCCTTCCAAAATAA      | TTATCTTCTCTCACACATACCTC (83) | TAGGTTTTGATGTTTATTTTAT          | F1-R                 | F2       |
| Neuromedin U                           | CAACATCCCTACAAAACACACTCT       | TCCATTCTCTTATCCCAA           | TTTATTTGGTGTGTTGGTTGTGTTT       | F1-R                 | F2       |
| TAP1                                   | AAACCCCAATAAAAATAATACCAATC     | TAAACACCTAATTTCCCAAC         | TGTTTAAATTTTATTTATGTATA         | F1-R                 | F2       |
| Lysosomal neuraminidase precursor      | CACAAAACCAATCCTTCTCCCAA        | CTTCTCCACCCCAACCAAT          | ATTTTGGTAGTTAGATTTTATAGAG       | F1-R                 | F2       |
| claudin-3                              | TTTGGTTAGTTTATAGGTGTAGT (98)   | TCAGTATCCTTAAACCCCTAC        | GGACGGATGGATGGATTGATTTAT        | F1-R                 | F2       |
| BMP-1                                  | AAAAATCATAAAAAAACCCGTAA        | CGACTAAACCCCAAAACAA (99)     | TTTGGTTAGTTTATAGGTGTAGT (100)   | F1-R                 | F2       |
| Uncoupling protein 2                   | CTTAAACCCCTAAACCTAAATTCAA      | CCCAATAAAAACTAACAA           | CGAGCGTGGATAGTTAATTTTAAGG       | F2-R                 | F2       |
| CRIP-1                                 | CCCAACTACGGCTCAAAAACGCTTAC     | ATCCCCAAATCCAAATCCT          | TATTTTGTGTTTGGATAT              | F2-R                 | F2       |
| Metallothionein 1G                     | AATACCCCTAAACCTAAATTCAT        | TACACTTAACCCATCTCCTA         | TTAAGCCGAGAAGGGAGAGGTAGT        | F2-R                 | F2       |
| Rad                                    | AAAACTAAATAATCTAAAAACCCC (113) | CTTCTCTCACACCTCAAAAC         | GAGGGGTTGGTATTTTATAGGAGG        | F1-R                 | F2       |
| Apolipoprotein C1                      | TAAATTAATAAACTTTAAAC           | TTATCAATCAACTACTCAAACTTA     | TATTTAGTATTAAATTTGAATTTTG (115) | F1-R                 | F2       |
| CLF-1                                  | CCCTAAATCCCAACCATCTCCCA        | CCAAATACATTCTCTAACTC         | AGAGTAGTAGTAGTAGGGGTAGTAA       | F2-R                 | F2       |
| FLRG                                   | ACAAATTTACACCTCCACACTAAA       | ATTTACCCCTATAAACAT           | AGGTTAGATTGGGAAGGGGAGGG         | F1-R                 | F2       |
| Putative cyclin G1 interacting protein | ATCCCCCTAAATAAACCCAC (125)     | TAAATAACCTCCCAATAC (126)     | GCGGTGATTTTGATATTATTTT (127)    | F1-R                 | F2       |
| Transglutaminase 2                     |                                |                              |                                 | F2-R                 | F2       |

\*SEQ ID NO: - numbered from 65 to 127, from left to right, top to bottom; representative SEQ ID NO: shown.

# HNSCC #011 cell line treated by 5aza-dC

TABLE 5

|          |        |                                                                              |
|----------|--------|------------------------------------------------------------------------------|
| 1776_at  | 10.9   | L24564 /FEATURE= /DEFINITION=HUMRAD Human Rad mRNA, complete cds             |
| 1902_at  | 5.562  | Source: Human excision repair protein (ERCC1) mRNA, complete cds, clone pcD  |
| 36953_at | 3.5132 | Drosophila melanogaster Mothers against dpp (Mad) gene(DPC4 gene), GenBan    |
| 34892_at | 2.4292 | DR4 homolog; contains a death domain similar to TNF receptor; TRAIL receptor |
| 1879_at  | 3.7    | M14949 Human R-ras gene, exons 2 through 6                                   |
| 1860_at  | 2.3    | U58334 Human Bcl2, p53 binding protein                                       |
| 2031_s_a | 2.9    | U03106 Human wild-type p53 activated fragment-1 (WAF1) mRNA                  |
| 595_at   | 3.648  | tumor necrosis factor alpha inducible protein                                |
| 35151_at | 2.4474 | DOC-1 related protein                                                        |
| 1668_s_a | 2.4375 | Source: Homo sapiens (clone g7) von Hippel-Lindau disease tumor suppressor   |
| 1814_at  | 3.3    | Homo sapiens mRNA for TGF-beta1R alpha                                       |
| 425_at   | 5.8    | X67325 H.sapiens p27 Mrna                                                    |
| 38374_at | 2.3    | AF050110 Homo sapiens TGFb inducible early protein and early growth respons  |
| 1736_at  | 2.2    | M62402 Human insulin-like growth factor binding protein 6 (IGFBP6) gene      |
| 39781_at | 3.7    | U20982 Human insulin-like growth factor binding protein 4 (IGFBP4) gene      |
| 32034_at | 3.1    | AF041259 H.sapiens breast cancer putative transcription factor (ZABC1) gene  |

# TABLE 6 HNSCC #013 cell line. treated by 5aza-dC + TSA

|            |        |                                                                          |
|------------|--------|--------------------------------------------------------------------------|
| 1879_at    | 4.8    | M14949 Human R-ras gene, exons 2 through 6                               |
| 41848_f_at | 6.4361 | Source: Human MDA-7 (mda-7) mRNA, complete cds.                          |
| 1454_at    | 2.3633 | Source: Homo sapiens mad protein homolog (hMAD-3) mRNA, complete cds.    |
| 31851_at   | 2.3171 | Source: Homo sapiens mRNA for candidate tumor suppressor involved in     |
| 38886_i_at | 2.2503 | Source: Homo sapiens putative tumor suppressor NOEY2 mRNA, complete cds. |
| 1211_s_at  | 2.1    | U84388 Human death domain containing protein CRADD mRNA                  |
| 33642_s_at | 11.7   | U17986 Human GABA/noradrenaline transporter mRNA                         |
| 1348_s_at  | 2.7    | S79219 metastasis-associated gene (human, highly metastatic lung         |
| 1788_s_at  | 2.1    | U48807 Human MAP kinase phosphatase (MKP2) mRNA                          |
| 38477_at   | 2.1    | S81752 DPH2L=candidate tumor suppressor gene (ovarian cancer)            |
| 34308_at   | 2.9    | U90551 Human histone 2A-like protein mRNA                                |
| 33352_at   | 3.3    | X57985 H.sapiens genes for Histones H2B.1 and H2A                        |
| 37196_at   | 2.4    | H.sapiens VE-cadherin mRNA                                               |
| 38994_at   | 2.6    | AF037989 H.sapiens STAT-induced STAT inhibitor-2 mRNA                    |

What is claimed is:

1. A method of identifying at least one epigenetically silenced gene associated with a cancer, comprising:

a) contacting an array of nucleotide sequences representative of a genome with nucleic acid molecules corresponding to RNA expressed in cancer cells contacted with at least one agent that reactivates expression of epigenetically silenced genes, under conditions suitable for selective hybridization of nucleic acid molecules to complementary nucleotide sequences of the array; and

b) detecting increased hybridization of nucleic acid molecules of the cancer cells contacted with the at least one agent to a subpopulation of nucleotide sequences of the array,

as compared to a level of hybridization, if any, of nucleic acid molecules corresponding to RNA expressed in the cancer cells to at least one nucleotide sequence of the subpopulation of nucleotide sequences, under said conditions,

whereby said increased selective hybridization identifies reactivated expression of an epigenetically silenced gene,

thereby identifying at least one epigenetically silenced gene associated with the cancer.

2. The method of claim 1, wherein the agent that reactivates expression of epigenetically silenced genes comprises a methyltransferase inhibitor, a histone deacetylase inhibitor, or a combination thereof.

3. The method of claim 2, wherein the methyltransferase inhibitor is 5-aza-2'-deoxycytidine.

4. The method of claim 2, wherein the histone deacetylase inhibitor is trichostatin A.

5. The method of claim 1, wherein the nucleic acid molecules corresponding to RNA comprise cRNA.

6. The method of claim 1, comprising contacting the array with nucleic acid molecules corresponding to RNA expressed in cancer cells contacted with 5-aza-2'-deoxycytidine, trichostatin A, or a combination thereof.
7. The method of claim 1, wherein the at least one epigenetically silenced gene comprises a nucleic acid molecule as set forth in Table 2, or a combination thereof.
8. The method of claim 1, wherein the at least one epigenetically silenced gene comprises a nucleic acid molecule comprising apolipoprotein D, neuromedin U, swisprosin-2, Hep27, KIF5C, keratin 14, transglutaminase 2, MUC1, interleukin-1 R2, crystallin alpha2, cysteine-rich protein with LIM (CRIP-1), Rad, HEM45, KLF6, follistatin related protein FLRG, XAP-5, Tbc1d1, cyclin G1 interacting protein, or a combination thereof.
9. The method of claim 1, wherein the at least one epigenetically silenced gene comprises apolipoprotein D, neuromedin U, swisprosin-2, cytokine-like factor-1 (CLF-1), CRIP-1, cellular retinol binding protein, metallothionein 1G, keratin 14, interleukin-1 receptor 2, crystallin alpha2, or a combination thereof.
10. The method of claim 1, wherein the at least one epigenetically silenced gene comprises a methylation silenced gene.
11. The method of claim 10, wherein the at least one methylation silenced gene comprises apolipoprotein D, neuromedin U, CLF-1, CRIP-1, claudin-3, uncoupling protein-2, metallothionein 1G, transglutaminase 2, apolipoprotein C1, or a combination thereof.
12. The method of claim 1, wherein the at least one epigenetically silenced gene comprises a tumor suppressor gene.

13. The method of claim 12, wherein the tumor suppressor gene comprises apolipoprotein D, neuromedin U, or CRIP-1.

14. The method of claim 12, wherein the tumor suppressor gene comprises neuromedin B, or receptor of G protein signaling 2 (RGS2).

15. The method of claim 1, wherein the cancer is an esophageal squamous cell carcinoma.

16. The method of claim 1, wherein the cancer is a head and neck squamous cell carcinoma.

17. A method for identifying a cell that exhibits or is predisposed to exhibiting unregulated growth, comprising detecting, in a test cell, epigenetic silencing of at least one gene comprising a nucleic acid molecule as set forth in Table 2, Table 5, Table 6, or a combination thereof, thereby identifying the test cell as a cell that exhibits or is predisposed to exhibiting unregulated growth.

18. The method of claim 17, wherein at least one gene comprises apolipoprotein D, neuromedin U, swisprosin-2, Hep27, KIF5C, keratin 14, transglutaminase 2, MUC1, interleukin-1 receptor 2, crystallin alpha2, cytokine-like factor-1, cysteine-rich protein with LIM (CRIP-1), Rad, HEM45, KLF6, follistatin related protein FLRG, XAP-5, Tbc1d1, cyclin G1 interacting protein, cellular retinol binding protein, metallothionein 1G, claudin-3, uncoupling protein-2, apolipoprotein C1, or a combination thereof.

19. The method of claim 17, wherein the cell exhibiting, or predisposed to exhibiting unregulated growth, is a neoplastic cell.

20. The method of claim 19, wherein the neoplastic cell is a premalignant cell.

21. The method of claim 19, wherein the neoplastic cell is a cancer cell.

22. The method of claim 21, wherein the cancer cell is an esophageal squamous cell carcinoma cell.

23. The method of claim 21, wherein the cancer cell is a head and neck squamous cell carcinoma cell.

24. The method of claim 17, wherein the epigenetic silencing comprises methylation silencing, said method comprising detecting methylation silencing.

25. The method of claim 24, wherein detecting methylation silencing comprises contacting a region comprising a 5' regulatory region of the nucleic acid molecule comprising the gene with a methylation sensitive restriction endonuclease, which cleaves a recognition site in the 5' regulatory region comprising a methylated cytosine residue of a CpG dinucleotide, whereby cleavage of the nucleic acid molecule is indicative of methylation silencing of the gene of the test cell.

26. The method of claim 25, wherein the methylation sensitive restriction endonuclease is Acc III, Ban I, BstN I, Msp I, or Xma I.

27. The method of claim 24, wherein detecting methylation silencing comprises contacting a region comprising a 5' regulatory region of the nucleic acid molecule comprising the gene with a methylation sensitive restriction endonuclease, which cleaves a recognition site in the 5' regulatory region comprising a methylated cytosine residue of a CpG dinucleotide, provided the cytosine residue of the CpG dinucleotide is unmethylated, whereby a lack of cleavage of the nucleic acid molecule is indicative of methylation silencing of the gene of the test cell.

28. The method of claim 27, wherein the methylation sensitive restriction endonuclease is Acc II, Ava I, BssH II, BstU I, Hpa II, or Not I.

29. The method of claim 24, wherein detecting methylation silencing comprises contacting a 5' regulatory region of the nucleic acid molecule comprising the gene of the test cell with a chemical reagent that selectively modifies either an unmethylated cytosine residue or a methylated cytosine residue, and detecting a product generated due to said contacting, wherein the product is indicative of methylation of a cytosine residue in a CpG dinucleotide of the gene, thereby detecting methylation silencing of the gene of the test cell.

30. The method of claim 29, wherein detecting the product comprises an electrophoresis method, a chromatography method, a mass spectrometry method, or a combination thereof.

31. The method of claim 29, wherein the chemical reagent is hydrazine, thereby producing a hydrazine treated 5' regulatory region of the gene,  
said method further comprising contacting the hydrazine treated 5' regulatory region with a reagent that cleaves hydrazine modified cytosine residues to generate a product comprising fragments of the nucleic acid molecule comprising the gene,  
separating the fragments according to molecular weight, and  
detecting a gap at a position known to contain a cytosine residue in the 5' regulatory region of the gene, wherein the gap is indicative of methylation of a cytosine residue in the CpG dinucleotide in the gene, thereby detecting methylation silencing of the gene of the test cell.

32. The method of claim 31, wherein the reagent that cleaves the hydrazine modified cytosine residue is piperidine.

33. The method of claim 29, wherein the chemical reagent comprises bisulfite ions, whereby unmethylated cytosine residues in the 5' regulatory region of the gene are converted to bisulfite modified cytosine residues,

said method further comprising exposing the bisulfite ion treated gene to alkaline conditions, whereby bisulfite modified cytosine residues are converted to uracil residues, and

detecting an amount or distribution of uracil residues in the 5' regulatory region of the bisulfite ion treated gene of the test cell,

wherein a decrease in the amount or distribution of uracil residues in the 5' regulatory region of gene from the test cell, as compared to the amount or distribution of uracil residues in a corresponding bisulfite ion treated unmethylated gene following exposure to alkaline conditions, is indicative of methylation of cytosine residues in CpG dinucleotides in the 5' regulatory region of the gene, thereby detecting methylation silencing of the gene of the test cell.

34. The method of claim 33, wherein detecting the amount or distribution of uracil residues comprises determining the nucleotide sequence of the bisulfite modified 5' regulatory region of the gene following exposure to alkaline conditions.

35. The method of claim 33, wherein detecting the amount or distribution of uracil residues comprises contacting the bisulfite ion treated gene sequence, following exposure to alkaline conditions, with an oligonucleotide that selectively hybridizes to the 5' region regulatory of the gene containing uracil residues, and  
detecting selective hybridization of the oligonucleotide.

36. The method of claim 35, wherein the oligonucleotide comprises a detectable label, and wherein detecting selective hybridization comprises detecting the label.

37. The method of claim 36, wherein the detectable label is a radioisotope, a paramagnetic isotope, a luminescent compound, a chemiluminescent compound, a fluorescent compound, a metal chelate, an enzyme, a substrate for an enzyme, a receptor, or a ligand for a receptor.

38. The method of claim 35, wherein the oligonucleotide is a substrate for a primer extension reaction, and wherein detecting selective hybridization comprises detecting a product of the primer extension reaction.

39. The method of claim 38, wherein the oligonucleotide has a nucleotide sequence as set forth in any of SEQ ID NOS:1 to 127.

40. The method of claim 33, wherein detecting the amount or distribution of uracil residues comprises

contacting the 5' regulatory region of the gene with an amplification primer pair comprising a forward primer and a reverse primer under conditions suitable for amplification, wherein at least one primer of the primer pair comprises an oligonucleotide that selectively hybridizes to a nucleotide sequence of the 5' regulatory region containing uracil residues,

whereby generation of an amplification product is indicative of methylation of cytosine residues in CpG dinucleotides in the 5' regulatory region of the gene, thereby detecting methylation silencing of the gene of the test cell.

41. The method of claim 40, wherein the amplification primer pair comprises a primer pair, comprising a forward primer and a reverse primer as set forth in SEQ ID NOS:1, 2, and 65 to 127.

42. The method of claim 33, wherein detecting the amount or distribution of uracil residues comprises

contacting the 5' regulatory region of the gene with an amplification primer pair comprising a forward primer and a reverse primer under conditions suitable for amplification, wherein both primers of the primer pair selectively hybridize to a nucleotide sequence of the 5' regulatory region containing cytosine residues, but not to a corresponding nucleotide sequence of the 5' regulatory region containing uracil residues, and

whereby generation of an amplification product is indicative of a lack of methylation of cytosine residues in CpG dinucleotides in the 5' regulatory region of the gene, thereby detecting methylation silencing of the gene of the test cell.

43. The method of claim 42, wherein the amplification primer pair comprises a primer pair as set forth in SEQ ID NOS:3 and 4.

44. The method of claim 33, wherein detecting the amount or distribution of uracil residues comprises

contacting in the 5' regulatory region of the gene with a first amplification primer pair and a second amplification primer pair under conditions suitable for amplification,

wherein the first amplification primer pair comprises a forward primer and a reverse primer, wherein at least one primer of the first primer pair comprises an oligonucleotide that selectively hybridizes to a nucleotide sequence of the 5' regulatory region of the gene containing uracil residues, and

wherein the second amplification primer pair comprises a forward primer and a reverse primer, wherein both primers of the second primer pair selectively hybridize to a nucleotide sequence of the 5' regulatory region of the gene containing cytosine residues, but not to a corresponding nucleotide sequence of the 5' regulatory region of the gene containing uracil residues, and

wherein an amplification product, if any, generated by the first primer pair has a first length, and wherein an amplification product, if any, generated by the second primer pair has a second length, which is different from the first length,

whereby the length of the amplification products is indicative of uracil residues and, therefore, methylation of cytosine residues in CpG dinucleotides in the 5' regulatory region of the gene, thereby detecting methylation silencing of the gene of the test cell.

45. The method of claim 24, wherein detecting methylation silencing comprises

a) contacting the test cell with a demethylating agent, and

b) detecting reactivated expression of an RNA encoded by the gene as compared to a level of expression of the RNA in a corresponding test cell not contacted with a demethylating agent.

46. The method of claim 45, wherein the demethylating agent comprises a methyltransferase inhibitor.

47. The method of claim 46, wherein the methyltransferase inhibitor comprises 5-aza-2'-deoxycytidine.

48. The method of claim 17, which is performed in a high throughput format, wherein the test cell, or extract of the test cell, comprises one of a plurality of test cells, or extracts of the test cells, or a combination thereof.

49. The method of claim 48, wherein each of the test cells, or extracts of the test cells, of the plurality is the same or different, or a combination thereof.

50. The method of claim 48, further comprising detecting methylation, if any, of cytosine residues in a CpG dinucleotide in a CpG island of the 5' regulatory region of the gene in a corresponding cell exhibiting regulated growth, or an extract of the corresponding cell.

51. The method of claim 48, wherein the test cells, or extracts of the test cell, are arranged in an array.

52. The method of claim 51, wherein the array is an addressable array.
53. The method of claim 48, wherein the test cells, or extracts of the test cells, are on a microchip, a glass slide, or a bead.
54. The method of claim 17, wherein the test cell comprises a sample obtained from a subject.
55. The method of claim 54, wherein the subject is a human subject.
56. The method of claim 54, wherein the sample comprises an organ sample, a tissue sample, or a cell sample.
57. The method of claim 56, wherein the sample comprises an alimentary tract sample, an esophageal sample, a liver sample, a skin sample, a lymph node sample, a kidney sample, a lung sample, a muscle sample, a bone sample, a gastrointestinal tract sample, or a brain sample.
58. The method of claim 56, wherein the sample comprises a biological fluid.
59. The method of claim 56, wherein the biological fluid comprises bone marrow, blood, serum, lymph, cerebrospinal fluid, saliva, sputum, stool, urine, or ejaculate.
60. A method of reducing or inhibiting unregulated growth of a cell exhibiting epigenetic silenced transcription of at least one gene associated with a cancer, the method comprising restoring expression of a polypeptide encoded by the epigenetic silenced gene in the cell, thereby reducing or inhibiting unregulated growth of the cell.
61. The method of claim 60, wherein restoring expression of the polypeptide comprises contacting the cell with a demethylating agent, a histone deacetylase inhibitor, or a combination thereof.

62. The method of claim 61, wherein the demethylating agent comprises a methyltransferase inhibitor.

63. The method of claim 60, wherein the epigenetic silenced gene comprises a methylation silenced gene, said method comprising contacting the cell with at least a demethylating agent.

64. The method of claim 63, wherein contacting the cell with the demethylating agent is performed in culture.

65. The method of claim 64, wherein contacting the cell with the demethylating agent comprises administering the agent to subject comprising the cell.

66. The method of claim 63, wherein the demethylating agent is 5-aza-2'-deoxycytidine.

67. The method of claim 60, wherein restoring expression of the polypeptide comprises introducing a polynucleotide encoding the polypeptide into the cell, whereby the polypeptide is expressed from the polynucleotide.

68. The method of claim 67, wherein the polynucleotide is contained in a vector.

69. The method of claim 68, wherein the vector is a viral vector.

70. The method of claim 67, wherein introducing the polynucleotide into the cell comprises contacting the cell with the polynucleotide *in vivo*.

71. The method of claim 67, wherein the epigenetic silenced gene comprises a nucleic acid molecule as set forth in Table 2.

72. The method of claim 60, wherein the epigenetic silenced gene comprises apolipoprotein D, neuromedin U, swisprosin-2, Hep27, KIF5C, keratin 14, transglutaminase 2, MUC1, interleukin-1 receptor 2, crystallin alpha2, cytokine-like factor-1 (CLF-1), cysteine-rich protein with LIM (CRIP-1), Rad, HEM45, KLF6, follistatin related protein FLRG, XAP-5, Tbc1d1, cyclin G1 interacting protein, cellular retinol binding protein, metallothionein 1G, claudin-3, uncoupling protein-2, apolipoprotein C1, or a combination thereof.

73. The method of claim 60, wherein the epigenetic silenced gene comprises a methylation silenced gene.

74. The method of claim 73, wherein the methylation silenced gene comprises apolipoprotein D, neuromedin U, CLF-1, CRIP-1, claudin-3, uncoupling protein-2, metallothionein 1G, transglutaminase 2, apolipoprotein C1, or a combination thereof.

75. The method of claim 60, wherein the epigenetic silenced gene comprises a tumor suppressor gene.

76. The method of claim 75, wherein the tumor suppressor gene comprises apolipoprotein D, neuromedin U, or CRIP-1.

77. The method of claim 75, wherein the tumor suppressor gene comprises neuromedin B, or receptor of G protein signaling 2 (RGS2).

78. A method for treating a cancer patient, wherein cancer cells in the patient exhibit epigenetic silenced expression of at least one gene, the method comprising restoring expression of the at least one epigenetic silenced gene in cancer cells in the subject, thereby treating the cancer patient.

79. The method of claim 78, wherein the at least one epigenetic silenced gene comprises a methylation silenced gene.

80. The method of claim 79, comprising administering a demethylating agent to the subject in an amount sufficient to restore expression of the methylation silenced gene in cancer cells in the subject.

81. The method of claim 78, comprising administering at least one polynucleotide encoding at least one polypeptide encoded by the at least one epigenetic silenced gene to the subject under conditions sufficient for expression of the at least one polypeptide in cancer cells in the subject.

82. The method of claim 81, wherein the polynucleotide is contained in a vector.

83. The method of claim 82, wherein the vector is a viral vector.

84. The method of claim 81, wherein the polynucleotide comprises a matrix.

85. The method of claim 84, wherein the matrix is a liposome.

86. The method of claim 78, wherein the cancer is carcinoma or a sarcoma.

87. The method of claim 78, wherein the cancer is an esophageal squamous cell carcinoma.

88. The method of claim 87, wherein the at least one epigenetic silenced gene comprises a nucleic acid molecule as set forth in Table 2.

89. The method of claim 87, wherein the epigenetic silenced gene comprises apolipoprotein D, neuromedin U, swisprosin-2, Hep27, KIF5C, keratin 14, transglutaminase 2, MUC1, interleukin-1 receptor 2, crystallin alpha2, cytokine-like factor-1 (CLF-1), cysteine-rich protein with LIM (CRIP-1), Rad, HEM45, KLF6, follistatin related protein FLRG, XAP-5, Tbc1d1, cyclin G1 interacting protein, cellular retinol

binding protein, metallothionein 1G, claudin-3, uncoupling protein-2, apolipoprotein C1, or a combination thereof.

90. The method of claim 87, wherein the epigenetic silenced gene comprises a methylation silenced gene.

91. The method of claim 90, wherein the methylation silenced gene comprises apolipoprotein D, neuromedin U, CLF-1, CRIP-1, claudin-3, uncoupling protein-2, metallothionein 1G, transglutaminase 2, apolipoprotein C1, or a combination thereof.

92. The method of claim 87, wherein the epigenetic silenced gene comprises a tumor suppressor gene.

93. The method of claim 92, wherein the tumor suppressor gene comprises apolipoprotein D, neuromedin U, or CRIP-1.

94. The method of claim 92, wherein the tumor suppressor gene comprises neuromedin B, or receptor of G protein signaling 2 (RGS2).

95. The method of claim 78, wherein the cancer is a head and neck squamous cell carcinoma.

96. The method of claim 95, wherein the at least one epigenetic silenced gene comprises a nucleic acid molecule as set forth in Table 5 or Table 6 or a combination thereof.

97. A method for selecting a therapeutic strategy for treating a cancer patient, comprising:

a) identifying at least one epigenetically silenced gene associated with the cancer by contacting an array of nucleotide sequences representative of a genome with nucleic acid molecules corresponding to RNA expressed in cancer cells contacted with at least one agent that reactivates expression of epigenetically silenced genes, under conditions suitable for selective hybridization of nucleic acid molecules to complementary nucleotide sequences of the array; and

detecting increased hybridization of nucleic acid molecules of the cancer cells contacted with the at least one agent to a subpopulation of nucleotide sequences of the array, as compared to a level of hybridization, if any, of nucleic acid molecules corresponding to RNA expressed in the cancer cells to at least one nucleotide sequence of the subpopulation of nucleotide sequences, under said conditions, whereby said increased selective hybridization identifies reactivated expression of an epigenetically silenced gene, thereby identifying at least one epigenetically silenced gene associated with the cancer; and

b) selecting an agent useful for restoring expression of the at least one epigenetically silenced gene in cancer cells of the patient, thereby selecting a therapeutic strategy for treating a cancer patient.

98. The method of claim 97, wherein the agent comprises a polynucleotide encoding the at least one epigenetically silenced gene.

99. The method of claim 98, wherein the polynucleotide comprises a nucleic acid molecule as set forth in Table 2, Table 5 or Table 6.

100. The method of claim 98, wherein the polynucleotide comprises apolipoprotein D, neuromedin U, swisprosin-2, Hep27, KIF5C, keratin 14, transglutaminase 2, MUC1, interleukin-1 receptor 2, crystallin alpha2, cytokine-like factor-1 (CLF-1), cysteine-rich protein with LIM (CRIP-1), Rad, HEM45, KLF6, follistatin related protein FLRG, XAP-5, Tbc1d1, cyclin G1 interacting protein, cellular retinol

binding protein, metallothionein 1G, claudin-3, uncoupling protein-2, apolipoprotein C1, or a combination thereof.

101. The method of claim 97, wherein the at least one epigenetic silenced gene comprises at least one methylation silenced gene.

102. The method of claim 101, wherein the agent comprises a polynucleotide comprising the at least one methylation silenced gene.

103. The method of claim 102, wherein the polynucleotide comprises apolipoprotein D, neuromedin U, CLF-1, CRIP-1, claudin-3, uncoupling protein-2, metallothionein 1G, transglutaminase 2, apolipoprotein C1, or a combination thereof.

104. The method of claim 101, wherein the agent comprises a demethylating agent.

105. The method of claim 104, wherein the demethylating agent is 5-aza-2'-deoxycytidine.

106. The method of claim 97, wherein the at least one epigenetically silenced gene comprises at least one tumor suppressor gene.

107. The method of claim 106, wherein the tumor suppressor gene comprises apolipoprotein D, neuromedin U, or CRIP-1.

108. The method of claim 106, wherein the tumor suppressor gene comprises neuromedin B, or receptor of G protein signaling 2 (RGS2).

109. A method of treating a subject suffering from an esophageal squamous cell carcinoma (ESCC), wherein cells associated with the ESCC contain at least one epigenetic silenced gene, comprising administering an amount of an agent that restores expression of the at least one epigenetic silenced gene to the subject sufficient to restore expression of the epigenetic silenced gene in cells associated with the ESCC, thereby treating the subject.

110. The method of claim 109, wherein the agent comprises a polynucleotide encoding the at least one epigenetically silenced gene.

111. The method of claim 110, wherein the polynucleotide comprises a nucleic acid molecule as set forth in Table 2.

112. The method of claim 110, wherein the polynucleotide comprises apolipoprotein D, neuromedin U, swisprosin-2, Hep27, KIF5C, keratin 14, transglutaminase 2, MUC1, interleukin-1 receptor 2, crystallin alpha2, cytokine-like factor-1 (CLF-1), cysteine-rich protein with LIM (CRIP-1), Rad, HEM45, KLF6, follistatin related protein FLRG, XAP-5, Tbc1d1, cyclin G1 interacting protein, cellular retinol binding protein, metallothionein 1G, claudin-3, uncoupling protein-2, apolipoprotein C1, or a combination thereof.

113. The method of claim 109, wherein the at least one epigenetic silenced gene comprises at least one methylation silenced gene.

114. The method of claim 113, wherein the agent comprises a polynucleotide comprising the at least one methylation silenced gene.

115. The method of claim 114, wherein the polynucleotide comprises apolipoprotein D, neuromedin U, CLF-1, CRIP-1, claudin-3, uncoupling protein-2, metallothionein 1G, transglutaminase 2, apolipoprotein C1, or a combination thereof.

116. The method of claim 109, wherein the at least one epigenetically silenced gene comprises at least one tumor suppressor gene.
117. The method of claim 116, wherein the tumor suppressor gene comprises apolipoprotein D, neuromedin U, or CRIP-1.
118. The method of claim 116, wherein the tumor suppressor gene comprises neuromedin B, or receptor of G protein signaling 2 (RGS2).
119. The method of claim 110, wherein the polynucleotide is contained in a vector.
120. The method of claim 119, wherein the vector is a viral vector.
121. The method of claim 112 wherein the polynucleotide comprises a matrix.
122. The method of claim 121, wherein the matrix is a liposome.
123. The method of claim 109, wherein the agent comprises a demethylating agent.
124. The method of claim 123, wherein the demethylating agent is 5-aza-2'-deoxycytidine.
125. The method of claim 109, wherein administering the agent comprises administering the agent to a site of the cells of the ESCC in the patient.
126. An isolated oligonucleotide, comprising an oligonucleotide as set forth in any of SEQ ID NOS:1 to 127.
127. A plurality of isolated oligonucleotides, comprising at least two of the isolated oligonucleotides of claim 126.

128. An amplification primer pair, comprising a forward primer and a reverse primer as set forth in SEQ ID NOS:1 to 127, wherein the amplification primer pair can amplify a portion of a nucleic acid molecule of Table 2.

129. The amplification primer pair of claim 128, which can specifically amplify a methylated 5' regulatory region of the nucleic acid molecule.

130. The amplification primer pair of claim 129, comprising a forward primer and a reverse primer as set forth in SEQ ID NOS:1, 2, and 65 to 127, which can specifically amplify a methylated 5' regulatory region of the nucleic acid molecule.

131. An amplification primer pair of claim 128, which can specifically amplify a unmethylated 5' regulatory region of the nucleic acid molecule.

132. The amplification primer pair of claim 131, comprising SEQ ID NOS:3 and 4.

133. A kit, which contains at least one isolated oligonucleotide of claim 126.

134. The kit of claim 133, which contains a plurality of isolated oligonucleotides.

135. The kit of claim 134, wherein said plurality comprises at least one amplification primer pair comprising a forward primer and a reverse primer.

136. The kit of claim 135, which contains a plurality of amplification primer pairs.

137. The kit of claim 135, wherein the amplification primer pair comprises a methylation specific amplification primer pair, an unmethylation specific amplification primer pair, or a combination comprising at least one methylation specific amplification primer pair and at least one unmethylation specific amplification primer pair.

138. The kit of claim 133, further comprising a reagent that modifies methylated cytosine residues.

139. The kit of claim 133, further comprising a methylation sensitive restriction endonuclease.

140. The kit of claim 133, further comprising reagents for performing an amplification reaction.

Microarray 12599 genes

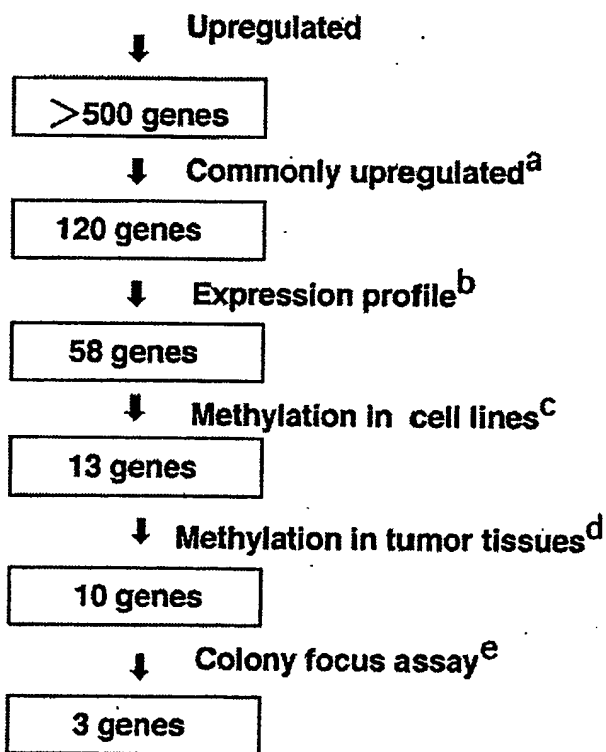


FIGURE 1

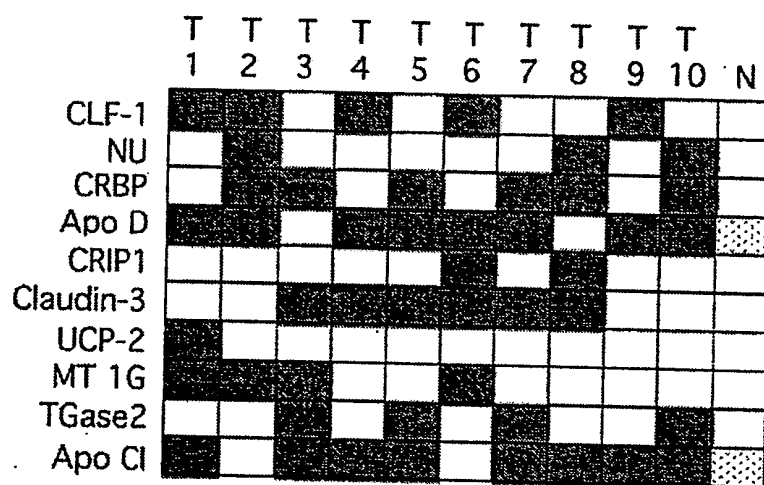


FIGURE 2

3/3

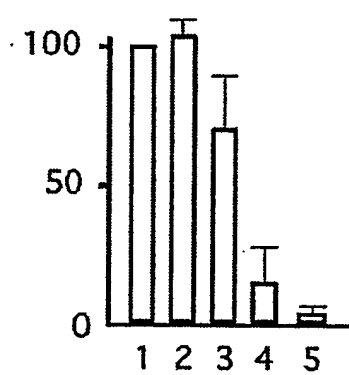


FIGURE 3

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